Regulation of immune responses by non--starch polysaccharides: Induction of distinct phenotypes in TLR--triggered dendritic cells and adjuvant properties

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Regulation of immune responses by non-starch polysaccharides: Induction of distinct phenotypes in TLR-triggered dendritic cells and adjuvant properties

Ph.D. thesis by

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ABSTRACT

Numerous non-starch polysaccharides (NSP) have shown immunoregulatory properties. The NSPs originate from both plant, fungal and microbial sources and constitute highly distinct structures. This present thesis focuses on comparing and identifying the immunomodulating capacities of these different NSPs using both *in vivo* and *in vitro* approaches.

The first study of this thesis addressed the importance of chemical structure, size, origin and presence of contaminants for the capacity of the NSPs to modulate the response pattern in dendritic cells (DC). Of the tested NSPs, especially the β-glucans and the galactomannan guar gum were found to modulate TLR4-triggered response profiles in murine bone marrow-derived DCs. Superior potency was demonstrated within the group of microbial-derived β-glucans, with cereal β-glucans and the galactomannan guar gum showing minor, but yet modulatory effects. In addition to the molecular structure of NSPs, their size may be of importance for the immunoregulatory properties, as comparisons of diverse cereal-based β-glucan products of different sizes varied in terms of their bioactivity.

The second study focused on a subgroup of NSPs; the β-glucans, and their ability to modulate the TLR4-triggered phenotype in human monocyte-derived DCs. The general phenotype was characterized by high-level expression of CCL4, IL-10, IL-2, IL-1β, IL-8, and TNF-α with low levels of CXCL10. Levels of IL-6, IL-12p70 and IL-23 varied dependent on the origin of the NSP. The C-type lectin receptors Dectin-1, DC-SIGN and the mannose receptor were down-regulated by the β-glucans, but to variable degrees dependent on the β-glucan origin. The overall DC signature demonstrates that β-glucans modify the TLR-induced phenotype in DC, and mediates a general fingerprint that implies induction of Treg- and/or Th17-promoting DC subsets.

The aim of the third study was to examine the *in vivo* adjuvant effect of the β-glucan lichenan on the antigen-specific response against the model protein β-lactoglobulin (BLG), specifically the importance of a complex formation between antigen and lichenan for the immunomodulating effect. In order to study this, a carbohydrate binding fusion protein of BLG and a carbohydrate binding module was constructed. The use of intraperitoneal injections into mice demonstrated that lichenan possessed adjuvant properties and induced an antigen-specific IgG response consisting of both IgG1 and IgG2a isotype antibodies, when non-covalently coupled to the antigen. In this *in vivo* system, lichenan showed to induce a mixed Th1/Th2-polarization of the antigen-specific response.

These results will help to provide a basic knowledge about polysaccharides, which are fundamental for achieving better understanding of their mechanisms of action. This will increase the possibility to develop and design new drugs that specifically maneuver the immune system against a desirable response. In addition this could add knowledge for development of food and food ingredients with specific nutritional and health properties.

DANSK RESUMÉ

Flere ikke-stivelses polysakkarider (NSP) har vist immunregulerende egenskaber. Disse stammer fra både plante-, svampe- og mikrobielle kilder, udgør vidt forskellige strukturer. Denne afhandling fokuserer på at sammenligne og identificere NSP´ernes immunmodulerende evner gennem både *in vivo* og *in vitro* studier.

Det første studie i denne afhandling undersøger betydningen af NSP´ernes kemiske struktur, størrelse, oprindelse og tilstedeværelsen af kontaminering for deres evne til at modulere dendritiske cellers (DC) immunrespons. Af de testede NSP´er, viste især β-glucaner og galactomannan guar gummi at kunne modulere en TLR4-medieret respons profil i murin knoglemarvs afledte DC´er. De mest potente blev fundet blandt β-glucaner fra mikrobielle kilder, β-glucaner fra korn og galactomannanen guar gummi var svagere, men stadig aktiv. Ud over NSP´ernes struktur, kunne deres størrelse også have betydning for deres immunregulerende effekt, eftersom en sammenligning af diverse kornbaserede β-glucan produkter af forskellige størrelse varierede med hensyn til deres bioaktivitet.

Den næste del fokuserede på at beskrive en undergruppe af NSP; β-glucaner, og deres evne til at modulere en TLR4-medieret responsprofil i human monocyte-afledte DC´er. Den generelle fænotype var karakteristisk ved et højt niveau af CCL4, IL-10, IL-2, IL-1β, IL-8 og TNF-α, samt et lavt niveau af CXCL10. Niveauer af IL-6 og IL-23 varierede afhængig af oprindelsen af NSP´en. C-type lectin receptorerne Dectin-1, DC-SIGN og mannose receptor udtrykket blev af alle β-glucaner nedreguleret, men i en varierende grad som var afhængig af typen af NSP´en. Den overordnede signatur af DC´erne, viser at β-glucanerne kan modificerer en TLR-induceret DC-fænotype og mediere et responsmønster der antyder at den inducerede DC subtype er Treg og/eller Th17 fremmende.

Formålet med det tredje studie var at undersøge *in vivo* adjuvant effekter af β-glucanen lichenan på et antigenspecifikt respons mod modelproteinet β-lactoglobulin (BLG), især betydning af en kompleksformation mellem antigen og lichenan. For at undersøge dette, blev et kulhydrat bindende fusionsprotein bestående af BLG og et kulhydrat bindende modul konstrueret. Ved brugen af intraperitoneale injektioner i mus, viste lichenan at besidde adjuvant-egenskaber og at kunne inducere et antigenspecifikt IgG respons, bestående af både IgG1 og IgG2a isotype antistofferne, når lichenan var koblet til antigenet. I dette *in vivo* system viste lichenan endvidere at inducere en blandet Th1/Th2 polarisation af det antigenspecifikke respons.

De opnåede resultater vil være med til at danne en basal viden omkring polysakkarider, som er fundamental for at kunne opnå en dybere forståelse of deres virkemåde. Dette vil styrke udviklingen og mulighederne for at designe nye medikamenter der specifikt vil kunne dirigere immunforsvaret mod et ønsket specifikt respons. Endvidere vil det også give muligheden for udviklingen af fødevare og levnedsmiddelingredienser med særlig ernærings- og sundhedsmæssige egenskaber.

PREFACE

The work included in the present Ph.D. thesis has been carried out at the Molecular Immune Regulation group, Center for Biological Sequencing at DTU Systems Biology, Technical University of Denmark in the period between January 2007 and August 2010. The thesis is a part of the Fibimmun project funded by the Danish Council for Strategic Research under the Programme Commision HEALTH, FOOD AND WELFARE.

Many people have contributed to this thesis and I wish to thank sincerely all the people who were involved, especially:

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René Wismar

Allerød, August 2010

ABBREVIATION

Ab	antibody
Ag	antigen
ANOVA	analysis of variance
APC	antigen-presenting cell
BBG	barley β-glucan
BLG	β-lactoglobulin
BMDC	bonemarrow-derived dendritic cell
CCR	chemotactic cytokine receptor
CBM	carbohydrate binding domain
CD	cluster of differentiation
CLR	C-type lectin receptor
CR3	Complement receptor 3
DCAL	dendritic cell associated C-type lectin
Dectin	DC-associated C-type lectin
DC	dendritic cell
DC-SIGN	DC-specific intercellular adhesion molecule 3-grabbing nonintegrin
Dm	dry-matter
DP	degree of polymerization
E.	*Escherichia*
ELISA	enzyme-linked immunosorbent assay
EU	endotoxin units
FACS	flow assisted cell sorting
FCS	foetal calf serum
GM-CSF	granulocyte macrophage colonystimulating factor
iDC	immature DC
IFN	interferon
Ig	immunoglobulin
IL	interleukin
Ip	intraperitoneal
LPS	lipopolysaccharide
Mϕ	macrophage
ManLAM	mannosylated lipoarabinomannans
MFI	mean fluorescence intensity
MICL	myeloid C-type lectin-like receptor
MINCL	macrophage-inducible C-type lectin
MHC	major histocompatibility complex
MoDC	monocyte derived dendritic cell
MR	mannose recepter
Mw	Molecular weight
NF-κB	nuclear factor κB
NO	nitric oxide
NOD	nucleotide-binding oligomerization domain
NSP	non-starch polysaccharides
OBG	oat β-glucan
P.	*Pichia*
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cell
PBS-T	phosphate-buffered saline (with Triton X-100)
PCA	principal component analysis
PMB	Polymyxin B
PRR	pattern recognition receptor
S.	*Staphylococcus*
SD	standard derivation
TCR	T cell receptor
TGF	tumor growth factor
Th	helper T cell
TLR	Toll-like receptor
TNF	tumour necrosis factor
Treg	regulatory T cel

TABLE OF CONTENT

CHAPTER 1

INTRODUCTION

This thesis chapter 1 and 2 introduces the theoretical framework for the experimental work. The aim of chapter 1 is to give a brief overview of the immune system, with focus on dendritic cells (DC) and their interaction with T and B cells. This section is based on murine studies unless stated otherwise.

Chapter 2 is a review of nonstarch-polysaccharides and their immunoregulatory activity both *in vivo* and *in vitro*. The review is entitled ”Dietary fibers as immunoregulatory compounds in health and disease” and is published in Annals of the New York Academy of Science in 2010.

THE IMMUNE SYSTEM

The immune system is a complex system that protects mammals from pathogens while at the same time maintaining self-tolerance. The system can be divided into two parts: the innate and the adaptive immune system. The innate system is non-specific and utilizes pattern recognition receptors (PRRs) to identify pathogens. Activation of an innate response causes inflammation, triggering of the complement system and the phagocytes, whose function is to destroy foreign material. The adaptive system is antigen-specific and involves the T and B lymphocytes. The immune response can either be a humoral or cell-mediated immune response. The humoral response is mediated by secretion of antigen-specific antibodies by B cells. A cell-mediated response involves cytotoxic T (Tc) cells that induce apoptosis of infected cells display foreign antigens on their surface.

DENDRITIC CELLS

DCs are one of the most important cell types of the immune system. The name is derived from their long dendrites protruding from the cell body that enables DCs to interact with antigens and other cells. DCs belong to the group of antigen presenting cells (APC) and their main function is antigen (Ag) uptake and presentation, which is an key step in the initiation and modulation of immune responses [1]. Immature DCs (iDC) circulates the blood and migrates to both tissue and lymph nodes. iDCs can be regarded as immune response sentinels, as they have a high endocytic activity and continuously sample the environment for soluble Ag, particles or apoptotic cells. The Ag

capture is the first crucial step for the induction of an immune response. DC uses several ways to capture Ag, such as macropinocytosis or receptor-mediated endocytosis. Upon Ag capture iDCs undergo phenotypic and functional changes. They reduce their Ag-capturing capacity and migrates to the T cell rich area of the secondary lymphoid tissue [2]. Simultaneously, DCs undergo a maturation process in which costimulatory molecules (CD40, CD80 and CD86) are up-regulated, MHC I and II is accumulated on their surface and the production of different cytokines is initiated [3]. These changes prepare the DC for the interaction with the T cells.

Several subtypes of DCs have been described both in humans and mice, based on their anatomic location and cell surface phenotypes. DCs in mice can be divided into myeloid DC (mDC) and plasmacytoid DC (pDC). Based on their expression of CD4, CD8α, CD11b and CD205 [4], mDCs can be divided into $CD8\alpha^{+}CD4^{-}CD11b^{-}CD205^{+}$, $CD8\alpha^{-}CD4^{+}CD11b^{+}CD205^{-}$, $CD8\alpha^{-}CD4^{-}CD11b^{+}CD205^{-}$, and $CD8\alpha^{-}CD4^{-}CD11b^{-}CD205^{-}$ subsets. All reside in both the lymph nodes and the spleen [4].

In this thesis DCs have been generated *in vitro* from either murine bone marrow stem cells (BMDC) or human peripheral blood mononuclear cells (PBMC). Comparison of *in vitro* generated murine BMDC with DCs isolated from the spleen and the mesenteric lymph node (MLN), has shown that spleen DCs is more similar to BMDC than the DCs isolated from MLN [5]. Splenic $CD8\alpha^{+}$ DCs are found to be the major producers of the IL-12 cytokines upon LPS stimulation. By this, $CD8\alpha^{+}$ DCs drive the development of a T helper cell (Th)-1 type immune response, whereas the $CD8\alpha^{-}$ DCs are more prone to induce a Th2-type response [6]. Monocyte-derived DCs (MoDC) from PBMC are comparable to blood DCs (BDC). However, *in vitro* generated MoDCs seem to be in a more mature state than BDCs [7].

DC AND INNATE IMMUNITY

The innate immune system is based of recognition of constitutively conserved molecules, often called pathogen-associated molecular pattern (PAMP). Receptors of the innate immune system that recognize PAMPs are the PRRs. The principal functions of PRRs includes: opsonization, activation of complement and coagulation cascades, phagocytosis, activation of pro-inflammatory signaling pathways and induction of apoptosis [8]. PRRs also induce differential signaling pathways and control DC maturation and subsequently the differentiation of Th cells. DCs is regarded as a link between the innate and the adaptive immune system [9]. Two of the PRR groups expressed on DC

are the Toll-like receptors (TLR) and C-type lectin receptors (CLR).

Toll-like receptors

The family of TLRs includes at least 10 receptors in humans and 13 in mice. TLR1-9 are conserved between humans and mice. TLR10 and 11 are only expressed in humans and mice, respectively [10]. TLR share the same intracellular signaling pathway, but recognizes different conserved structures on microorganisms. TLR are primarily expressed on cells of the innate immune system, enabling a fast initiation of an immune response toward foreign microorganisms [11]. PAMPs for murine have so far been identified for TLR1-9 and 11 (Table 1).

Table 1. Mouse toll-like receptors: TLR ligands and their presence on microorganisms.

TLR	Ligand	Microorganism	Ref.
1/2	Triacyl lipopeptides	Bacteria	[12]
2	Peptidoglycans, phospholipomannans	Gram positive bacteria, fungi	[13]
2/6	Lipoteichoic acid, diacyl lipopeptides	Bacteria	[14]
3	Dobbelt stranded RNA, Poly (I:C)	Virus	[15]
4	LPS, mannan	Gram negative bacteria, Fungi	[16]
5	Flagellin	Flagellated bacteria	[17]
7/8	Single stranden RNA	Virus	[18]
9	CpG DNA	Bacteria, Virus	[19]
11	Profilin	Fungi	[20]

TLRs are either present on the cell membrane (TLR1, 2, 4, 5, 6 and 11) or intracellularly (TLR3, 7, 8, 9). Triggering of the TLR induces production of pro-inflammatory cytokines via the transcription factor nuclear factor κB (NF-κB) or by inducing production of type I IFNs [21].

C-type lectin receptors

The hallmark of CLRs are their Ca^{2+}-dependent carbohydrate recognition domains [22]. A small group of CLRs are however Ca^{2+}-independent, but still very functionally similar, and therefore named C-type lectin-like receptors. CLRs play an important role in binding and uptake of microbial components [23]. CLRs interact with pathogens through the recognition of glycan structures. The CLRs; DC-specific ICAM-3 grapping non-intergrin (DC-SIGN), mannose receptor (MR), Langerin and macrophage galactose lectin receptor (MGL) recognize high mannose (typically consisting of five to nine mannose units), fucose, GalNAc or GlcNAc in a Ca^{2+}-dependent way [24, 25]. DC-associated C-type lectin 2 (Dectin-2) and macrophage-inducible C-type lectin (Mincle) both bind mannose [26, 27]. Dectin 1 the most well described receptor for β-glucan [28]. Not all CLR ligands

are known and myeloid C-type lectin-like receptor (MICL) and dendritic cell associated C-type lectin 2 (DCAL-2) are less described and the ligands are presently unknow.

INTERACTION OF DCS WITH OTHER CELLS

Upon capturing of antigen, DCs migrate towards the lymph vessels and are carried via the lymph flow to the draining lymph nodes. The lymph nodes become a collection point where DCs can interact with T and B cells to initiate and shape the adaptive immune response. DCs navigate by chemotaxis, which involves interacting with chemokines that are expressed on the surface of cells or have been released as chemical messengers to draw DCs to the lymph nodes. Enzymes within the DCs digest the captured antigen into smaller pieces containing epitopes, which are then presented to T cells using MHC. DCs do not only present its cargo to the T cell, it also communicates via cytokines and co-stimulatory molecules on the surface, which lead to differentiation of the T cell. This section focuses on the interaction of DCs with T cells and B cells.

Interaction with T cells

A naïve T cell has undergone both the positive and negative selection in the thymus. Naïve T cells are regarded as mature but they have not encountered any Ag. Two types of T cell subset exist: $CD4^+$ and $CD8^+$ T cells. $CD8^+$ T cells primarily recognize peptides processed from endogenously synthesized Ag presented by MHC class I molecules. After activation, they differentiate into effector cells producing interferon (IFN)-γ and tumor necrosis factor (TNF)–α and some become cytotoxic (via perforin and granzyme B) [29]. The primary role of $CD8^+$ T cell is to proliferate and protect against viral infections by killing infected cells [30]. After clearance of the infection, approximately 10% persist as memory $CD8^+$cells [31]. $CD4^+$ Th recognizes peptides presented by the MHC class II molecules on APC. Upon activation naïve T cells differentiate into separate functional effector subsets specialized in producing cytokines in different combinations: Th1, Th2, Th17 and regulatory T (Treg) cells. The major cytokines involved in Th cell differentiation are: IL-6, IL-10, IL-12p70, IL-23, IFN-γ and transforming growth factor (TGF)-β. Figure 1 illustrates the cytokines involved in differentiation of the different T cells.

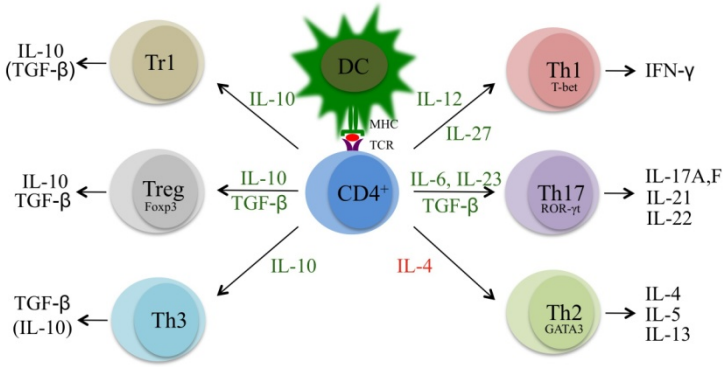

Figure 1. Differentiation of naïve CD4$^+$ T cells.
Certain DCs derived cytokines (in green) and cytokines from other sources (in red) mediate differentiation of naïve CD4$^+$ T cells into several subsets of T helper cells (Th1, Th2 or Th17) or regulatory subsets (Treg, Tr1 or Th3). Distinctive maturation pattern in DCs result in different cytokine profiles that mediates the differentiation of T cell subsets. The different T cell subsets express certain transcription factors and produce specific lineage dependent cytokines**.**

Th1 cells produce high levels of IFN-γ and are important in the immune defense against intracellular bacteria and tumors. Th1 cells are highly pro-inflammatory and have been linked to many autoimmune diseases [32-34]. The key cytokine for skewing the differentiation of Th1 cells is the heterodimeric cytokine IL-12p70 (p40 and p35) through the IL-12 receptor present on the naïve T cell. Activation of the IL-12 receptor induces the transcription factor STAT4, which promotes expression of multiple Th1-cell associated genes, including the *Ifnγ* gene that leads to production of IFN-γ [35]. The release of IFN-γ initiate an auto/paracrine positive feedback loop, in which activation of the IFN-γ receptor promotes expression of the *Tbx21* gene that encodes the transcriptions factor T-bet. T-bet induces both the *Ifnγ* gene and promotes expression of the IL-12 receptor β2 chain. This all together increases the T cell responsiveness towards IL-12p70 [35]. IL-27 has also been connected to Th1 differentiation. IL-27 induces Th1 differentiation through the intercellular-adhesion molecule (ICAM)/ lymphocyte function-associated antigen 1 (LFA1) pathway [36].

Th2 cells promote B cell proliferation and plasma cell development and thereby support antibody production (the humoral immune response). No DC-derived cytokine has directly been connected to differentiation of Th2 cells. Th2 differentiation is initiated by TCR signaling in concert with IL-4 receptor signaling via STAT6. These signals up-regulates GATA-3, an important regulator of Th2 differentiation [37, 38]. Initial TCR signaling induces low level expression of both GATA-3 and IL-4. GATA-3 enhances the transcription of the *Il4*, *Il5* and *Il13* genes. The increased IL-4 further

increases the Th2 cell differentiation in a feed forward loop [39]. IL-9 is also regarded as a Th2 produced cytokine. However, IL-9 seems to be regulated in another way than the other Th2 cytokines. The regulation of IL-9 is not known, and the IL-9 producing T cell may be of a new subset (Th9) that is Th2 related [40].

Both Th1 and Th2 regulation can reinforce their own development through positive feedback; however they also cross-regulate each other's differentiation by negative feedback. T-bet that promotes Th1 cell differentiation prevents Th2 differentiation by inhibiting GATA-3. GATA-3 prevents the Th1 differentiation by inhibiting expression of both the IL-12 receptor β2 chain and the *stat4* gene [6, 41].

Besides differentiation of $CD4^+$ cells into the two effector subsets Th1 or Th2, $CD4^+$ T cells can also differentiate into distinct regulatory subsets. This subset of cells is characterized by their ability to engage in the maintenance of immunological self-tolerance and immune homeostasis [42]. One class of the regulatory T cells is the natural Tregs (nTregs) that develops intrathymically. nTregs cells are $CD4^+Foxp3^+$ and dependent on IL-2 and TGF-β for maintenance [43]. nTregs may mediate its suppressor function through the cytotoxic T lymphocyte-accosiated-4 (CTLA-4) that binds to CD80/CD86 on DC and induces immunosuppressive signals [44]. Another class of Tregs that develops from naïve $CD4^+$ T cell precursors in the periphery is the induced or adaptive Tregs (iTregs). The $Foxp3^+$ iTregs need IL-2 and TGF-β for generation and maintenance and mediates their effects via production of TGF-β and IL-10 [45, 46]. Two subsets of $Foxp3^-$ iTreg have been described: Th3 and Tr1 cells. Both are induced by IL-10 and produce both IL-10 and TGF-β. It is possible to distinguish the two subtypes given that Tr1 produces mainly IL-10 and Th3 TGF-β [47, 48]. iTreg mediates their effect via secretion of inhibitory cytokine. IL-10 has been shown to inhibit IL-12 production in APCs and may therefore indirectly lead to the inhibition of IFN-γ, and consequently Th1 differentiation [49].

Recently, a distinct lineage of Th cells, the Th17 was identified and characterized [50]. This cellular Th subset is named after their main cytokines produced: IL-17A and IL-17F. Th17 cells also produce the cytokines IL-21 and IL-22 [51]. The differentiation of murine Th17 is mediated by cytokines and growth factors including: TGF-β, and IL-6 while human Th17-polarization requires IL-1β and IL-6. IL-23 is needed as a growth and stabilization factor [50, 52]. Th17 cells have been

associated with host defense against extracellular pathogens and are involved in several autoimmune diseases [53].

Interaction through costimulatory molecules

In addition to the DC and T cell derived cytokines, interactions between co-stimulatory molecules on DCs and T cells in the immunological synapse, have influence on the programming of T cells and the initiation of an adaptive immune response (figure 2). The activation of naive T cells and their differentiation into effector T cells requires most often the generation of two signals. Signal 1 is generated after the interaction of the T cell receptor (TCR) with the antigen bearing MHC on APCs. Co-stimulation signal delivers the 2nd signal. CD28 is probably the most important, as it enhances TCR-induced proliferation and differentiation of naive T cells. CD28 interaction with CD80/86 up-regulates the expression of cytokines, chemokines, receptors for cytokines and chemokines and other co-stimulatory receptors [54]. Amongst the up-regulated receptors are CD40. CD40 acts as an activation stimulus of DC and up-regulates CD80, CD86, IL-12 secretion and chemokine production [55, 56]. CD40-CD40L ligation activates $CD4^+$- and $CD8^+$ T cells [57]. The CTLA-4 receptor competes with CD28 for interaction with CD80/86. Unlike CD28, CTLA-4 inhibits the immune responses and is a critical mediator of peripheral tolerance [58]. On immature DCs, CD86 is expressed at low levels, and CD80 even lower, and upon maturation, CD86 is up-regulated more rapidly than CD80. The exact significance of CD80 and CD86 on the differentiation of naïve T cells is presently not clear [59].

Activation of the OX40 receptor on T cells by the ligand OX40L present on DCs, enhances cytokine production and proliferation of T cells. OX40 does not seem to directly contribute in the differentiation of a response into Th1 or Th2, rather it adds to further activation of ongoing Th1 or Th2 responses, and may have greater effects on Th2 responses due to the higher levels of OX40 on Th2 cells [60].

The receptor inducible costimulator (ICOS) is poorly expressed or absent on naïve T cell, although ICOS is present on activated T cells. ICOS is expressed on different Th cell subsets and play a role for their differentiation. Th17 cells however only need ICOS for their expansion [61, 62]. The ICOS ligand (ICOS-L) is expressed on several types of APC [63]. ICOS-L is present on pDC and at low levels on myeloid DC. Ligation of ICOS-L on pDC leads to the differentiation of IL-10-producing Treg cells [64] while immature myeloid DC induces T cell anergy [65]. However ICOS/ICOS-L has

also been shown to induce Th2 differentation [63].

The notch pathways are highly conserved in cell-to-cell communication and are important in the regulation of naïve T cell differentiation. Delta and Jagged are notch ligands expressed on DCs. The notch 3 ligand Delta 1 induces IFN-γ, which is linked to Th1 cells differentiation [66]. Jagged 1 has been related to Th2 differentiation due to the induced IL-4 production in T-cells upon ligation [67].

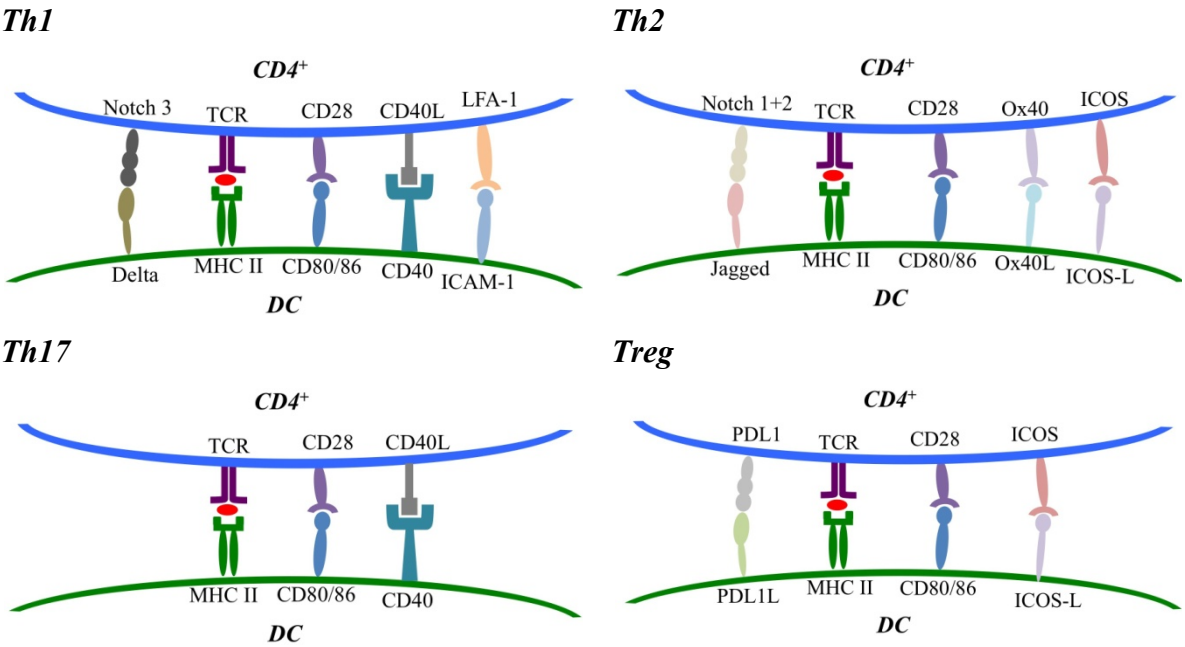

Figure 2. DC and T cell surface molecules in differentiation of naïve CD4$^+$ T cells.
Differentiation of naïve CD4$^+$ T cells into different subsets of T helper cells (Th1, Th2 or Th17) or regulatory subsets (Treg) is dependent on different co-stimulatory signals. These signals are generated by the interaction between surface receptors on DC and naïve CD4$^+$ T cells.

The pathway of programmed death ligand-1 (PD-L1) and PD-1 has a pivotal role in regulating iTreg cell development and in sustaining iTreg cell function. Murine APC (DC, macrophages and B cells) constitutively express PD-L1, while PD-1 is up-regulated on T cells upon activation [68].

Interaction with B cells

B cells are lymphocytes that take part in the humoral immune response. Their main functions are to act as APC, make antibodies and eventually develop into memory B cells after activation due to antigen interaction. B cells are, therefore, an essential component of the adaptive immune system.

Besides activating naïve T cells, DCs activate both naïve and memory B cells. Myeloid DCs have

been shown to trigger B cell growth and differentiation through the release of soluble factors such as IL-12 and IL-6 [69]. Viral-activated pDC have been shown to trigger CD40-activated B cells to differentiate into plasma cells. IFN-αβ generates non-Ig-secreting plasma blasts and IL-6 induces their differentiation into Ig-secreting plasma cell [70]. The B-cell activating factor (BAFF) plays a role for the survival of B cells. BAFF is expressed by monocytes, macrophages and DCs. Several cytokines such as interferon (IFN) α and γ, IL-10, granulocyte colony-stimulating factor and CD40L as well as lipopolysaccharide (LPS) and peptidoglycans can activate BAFF production [71].

CHAPTER 2

DIETARY FIBERS AS IMMUNOREGULATORY COMPOUNDS IN HEALTH AND DISEASE

In collaboration with Susanne Brix, Hanne Frøkiær and Helle Nygaard Lærke
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Many non-starch polysaccharides (NSPs) including NSPs classified as dietary fibers have been reported to possess immunoregulatory properties. The fibers reported to be able to activate or by other means modulate immune responses originate from both plant, fungal and microbial sources and constitute highly distinct structures. In order to obtain a better understanding of factors important for the immunoregulatory activities, this paper addresses the importance of chemical structure, origin and purity of fibers for their capacity to interact with key regulatory cells of the immune system. Furthermore, we assess the bioavailability, and discuss the possible mechanisms involved in intestinal absorption of NSPs.

The binding of some NSPs to carbohydrate receptors on immune cells is well-established and this event leads to activation or other changes in dendritic cells and macrophages. Especially β-glucans and some mannans have demonstrated immunomodulatory activities, but the specific structure is of major importance for the activity. Within β-glucans, branched β-(1,3)-D-/β-(1,6)-glucopyranosyl polymers from fungi exhibit far the strongest activity, but it varies according to structure, molecular weight, and solubility. As many of the preparations tested constitute crude extracts or only partly purified NSPs, the risk of contaminants holding immunoregulatory activities should not be ignored. Moreover, the use of different *in vitro* and *in vivo* approaches hampers direct comparison of immunomodulatory effects of different fibers.

To what extent the NSPs enter systemic circulation and are transported to remote sites of the body has been difficult to assess, partly due to lack of sensitive analytical methods. The presence of NSPs in blood and Peyer's patches in the gut has been demonstrated in few studies, supporting encounter between NSPs and immune cells, but bioavailability studies still constitute a major challenge. Studies demonstrating *in vivo* effects of β-glucans on microbial infections and cancer treatment strongly indicate an immunoregulatory mechanism behind the effects. However, the potential of NSPs as immuneregulatory food ingredients is still far from fully explored.

INTRODUCTION

A substantial number of reports on dietary fibers from a variety of plant crops and other non-starch polysaccharides (NSP) from algae, fungi and microorganisms, their interaction with cells of the immune system, and the cellular or physiological consequences of such interactions have emerged during recent years. NSPs form important parts of e.g. the cell wall of yeasts and fungi, while a range of bacteria produce extracellular polysaccharides [72] that are important components of their virulence in animals and/or plants. It is well-known that these structures are recognized by specific receptors on various immune cells. It is thus highly relevant to presume similar effects of NSP of other origins. All together, this has demonstrated interesting potentials of natural carbohydrate macromolecules, of which some are already present in foods or used as food ingredients, applied in biomedicine and functional foods.

Accordingly, a broad variety of carbohydrate structures in different combinations, with different physical-chemical properties, and of varying purity are being reported to hold immuno-stimulatory or in other ways immunomodulatory activities. *In vitro* assays using different cell types and cells of different origin, together with *in vivo* experiments based on various animal species, have been used to study the immunomodulating effects, and this has further added complexity to the current state-of-the-art. Accordingly, at the present state, some important questions have to be answered in order to unravel the actual potential of NSP of botanical and microbial origin. This includes questions regarding importance of the purity, structure, size and other physiochemical properties for the immunomodulatory activity, but also questions addressing whether the fibers or degradation products hereof are absorbed in significant amounts to stimulate the immune cells, or whether the effects take place by fiber-cell interaction in the gastrointestinal tract. Immunoregulatory effects of NSPs may arise from both direct modulations of immunocompetent cells via specific receptor-mediated activation or indirectly due to influences of NSPs on the indigenous microflora, which also is capable of affecting immune cells directly. The current focus is on direct effects of NSPs on immune cells.

We here address the importance of chemical structure, origin, purity and bioavailability of fibers from plants and microorganisms for their putative immunomodulating properties and hence their potential in functional food and biomedicine.

SPECIFICITY OF CARBOHYDRATE-BINDING RECEPTORS ON MACROPHAGES AND DCS

To interact with and activate or regulate immune responses in immune cells, NSPs need to hold specific molecular structures that bind to surface receptors on immune cells leading to induction of intracellular signal transduction pathways. Especially macrophages and dendritic cells (DCs) display carbohydrate-binding receptors and, as these cells are highly efficient antigen presenting cells, they play a key role in orchestrating adaptive immunity towards e.g. pathogens. The strategic location of macrophages and DCs at gut mucosal surfaces further strengthens the cells possibilities to sense and respond to food-administrated NSPs. The pattern recognition receptors (PRRs) on macrophages and DCs with carbohydrate-binding properties (Fig. 1), all excert high specificity for clustered glycans [73, 74]. The C-type lectins (CLRs) such as MR, DC-SIGN, MGL and langerin contain carbohydrate-binding domains that display Ca^{2+} -dependent binding to terminal high-mannose (typically consisting of five to nine terminal mannose units), fucose-containing glycans, GalNAc or GlcNAc [24, 25]. Dectin-1 is a Ca^{2+}-independent C-type lectin-like PRR and is currently the most well described receptor for (1,3)-β-glucans [28]. It binds to a wide variety of (1,3)-β-glucans containing a minimum of 9 units, and with affinities depending on the type and amount of side-chain branching [75, 76]. Complement receptor 3 (CR3), also known as (CD11b/CD18, Mac-1 or $\alpha_M\beta_2$ integrin) is the receptor for complement (C3b)-opsonized particles as well as β-glucan and various other ligands [77]. Polysaccharides that bind to CR3 either contain mannose, N-acetyl-D-glucosamine or glucose [78]. The ligands for Dectin-2, DCIR, Mincle and DCAL-2 are all less well described, although mannose has been ascribed to bind to dectin-2 [27], and to DCIR [79]. Mincle was recently reported to bind to *C. albicans*, but its glycan specificity is not elucidated yet [80].

Many reports have recently provided evidence that some of these CLRs exert their action by modifying the immune response programs induced by other activated PRRs such as the Toll-like receptors (TLR) [81, 82]. One common feature of some of the carbohydrate-binding PRRs, like DC-SIGN and MR, is their ability to affect the expression of specific cytokine genes that are generally induced after TLR activation [83, 84]. Specifically they do this by down-regulation of TLR-induced production of the pro-inflammatory cytokine IL-12p70 while enhancing the anti-inflammatory cytokine IL-10 in a synergistically manner [85]. In this sense, the carbohydrate-

binding PRRs diverge from other groups of PRRs, namely TLRs and nucleotide-binding oligomerization domains (NODs), which in general are more potent inducers of IL-12p70 leading to enhanced activation of cell-mediated immunity [10, 86]. So, currently our conception of immunoregulation by NSPs in DCs and possibly also in macrophages is based on the structural capacity of certain polysaccharide structures to modulate pro-inflammatory responses via interaction with these carbohydrate-binding PRRs. We therefore find it appropriate to use the term immunomodulation' to describe the immunological effects of NSPs, rather than immunostimulation, as one of the principal effects of the carbohydrate-binding PRRs that are known to bind exogenous glycan structures, is to modulate cell-mediated immune responses. Yet, we lack thorough insight into which NSP structures that most efficiently bind to and induce cellular signaling through these PRRs.

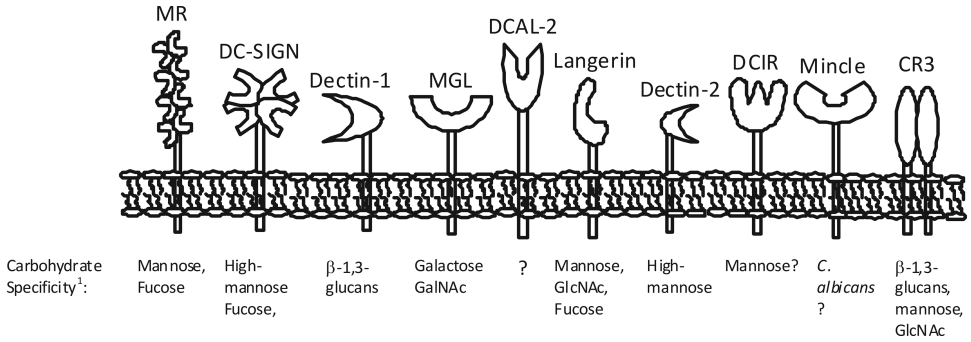

Figure 3. Carbohydrate-binding receptors on macrophages and dendritic cells.
Various C-type lectin receptors and also the pleiotropic receptor CR3contain one or more carbohydrate-binding domains with specificity for several different exogenous glycan structures. Abbreviations: MR: Mannose receptor, DC-SIGN: Dendritic Cell-Specific Intercellular adhesion molecule 3-Grabbing Nonintegrin, DCAL-2: DC-associated lectin-2, DCIR: DC immunoreceptor, Mincle: macrophage-inducible C-type lectin, Dectin: DC-associated C-type lectin, MGL: macrophage galactose specific lectin. CR3: Complement receptor 3. ?: The ligand is not known presently.

BIOACTIVE NSPS – STRUCTURE/FUNCTION RELATIONSHIP

There is a great variability among the effectiveness of NSPs as immunomodulators. One reason might be explained by their variety in chemical composition. But even within a group of chemically similar polysaccharides there are differences.

One group of polymers that are all composed of β-linked glucose units is β-glucans. β-glucans can be found in different sources such as: fungal cells walls, algae, bacteria and cereals. The β-glucans differ in structure (Table 1) depending of their origin, and this may explain why they do not have the same ability to stimulate the immune system. Our own results have shown that β-glucans from

microbial sources exhibit a stronger capacity to modulate the immune response in lipopolysaccharide (LPS)-stimulated DCs, compared to β-glucans from cereals, but even within β-glucans of microbial origin, the immunemodulating activity varies substantially (unpublished observations, manuscript in preparation). This finding is in agreement with a recent report describing that binding affinities of dectin-1 to various β-glucan structures vary according to the side-branching frequency as well as the chain length [75].

Table 1: Examples of structures of β-glucans from different sources.

Source	Structure	Common name
Bacteria *Alcaligenes faecalis*	Linear polymer of β-(1,3)-D-glucopyranosyl units	Curdlan
Euglenoids *Euglena gracilis*	Linear polymer of β-(1,3)-D-glucopyranosyl units	Paramylon
Fungi *Saccharomyces cerevisiae* *Lentinula edodes* *Sclerotinia sclerotiorum*	Polymer of β-(1,3)-D-glucopyranosyl units with branches of β-(1,6)-D-glucopyranosyl units	Zymosan Lentinan Scleroglucan
Algea *Laminaria digitata*	Polymer of β-(1,3)-D-glucopyranosyl units with branches of β-(1,6)-D-glucopyranosyl units	Laminarin
Cereal *Avena sativa* *Hordeum vulgare*	Linear polymer of mixed β-(1,3)-D-glucopyranosyl/β-(1,4)-D-glucopyranosyl units	Oat β-glucan Barley β-glucan
Lichen *Cetraria islandica*	Linear polymer of mixed β-(1,3)-D-glucopyranosyl/β-(1,4)-D-glucopyranosyl units	Lichenan

Other factors than their chemical and structural composition may play a role for β-glucan-associated biological activity. The molecular weight, solubility and helical conformations of the polysaccharides may also be important, and may actually depend on molecular structure. Yeast (1,3),(1,6)-β-D-glucans are insoluble [87, 88], but the solubility increases as the degree of polymerization of the (1,3)-β-glucan is lowered [89]. Soluble fungal β-glucans appear to be stronger immunostimulators than insoluble structures [90]. Fungal β-glucans can in their native form adopt either a single or a triple helical conformation, where hydrogen bonds hold the individual polymers together. These bonds can be interrupted by increased temperature, high pH, or several solvents [91]. Sonication, which reduces molecular weight, and chemical derivatization are other means to improve solubility [88]. Scleroglucan, an exopolysaccharide obtained from the filamentous fungus *Sclerotium*, is a (1,3)-β-D-glucan with branches of a single β-(1,6)-glucopyranose on every third backbone residue. In contrast to the yeast derived (1,3),(1,6)-β-D-glucans, the polysaccharide

dissolves in aqueous solution with a triple helical conformation stabilized by hydrogen bonds internally in the triple helical structure [92]. Laminarin, another (1,3)-D-glucan with branches of a single β-(1,6)-glucopyranose on approximately every tenth backbone residue – depending on source - is a storage polysaccharide of many species of sea weeds [93], which also displays triple-helix conformation. While laminarin in its original form in many studies fails to show immune modulatory effects [94], it has been shown that also β-glucan oligomers of laminarin obtained by enzymatic degradation have immune modulatory functions[93]. To what extent the triple helical conformation, the molecular weight, and the degree of branching are important to stimulate the immune system is still debated [92, 95]. The mixed-linked (1,3),(1,4)-β-D-glucans from cereals are also partly soluble, as the presence of the β-(1,3)-linkages in the linear polysaccharides prevents close packing of the molecule. These β-glucans have no single β-(1,4)-glucopyranose units, no repetitive β-(1,3)-glucopyranose units, and no branching, and this may explain why cereal β-glucans appear to be less potent biological response modifiers. However, recent studies indicate that lichenan, which is another mixed-linked (1,3),(1,4)-β-D-glucan along with other lichen derived polysaccharides (galactomannans and heteroglucans) show immunomodulatory effects in studies on DC maturation [96].

Mannans of the cell walls of yeast have a backbone of α-(1,6)-linked mannopyranose units with α-(1,2)-linked and α-(1,3)-linked side chains [97], and the effect of mannans of microbial origin to affect immune responses in macrophages and DCs has been described in numerous studies [98].

N-linked mannans, but not O-linked or phospo-mannans, have just recently been demonstrated to bind specifically to DC-SIGN and MR [99], thus suggesting that the specific structural organization of glycans is indeed important for PRR binding specificities. Due to their specific binding affinity, the mannans are presumably strong contributors to the immunomodulatory effect of zymosan and other yeast products. Other fungi contain α-linked mannans of varying structure. Pure mannans are uncommon in higher plants, but the leafs of aloe vera contain large amounts of a β-(1,4)-linked acetylated mannan (acemannan), which is known to enhance immunity and reduce oxidative injury [100-102]. Im *et al.* [103] showed that molecular size affected the immuno-modulatory activity. Galactomannan/heteroglycan polysaccharides of lichens were also recently shown to influence the cytokine production in monocyte-derived DCs [96, 104]. Again it was shown that differences in activity could be related to varying composition. Although galactomannans from guar to our

knowledge not earlier have been reported to affect the immune response, we have observed quite strong immunomodulating effects in DCs (unpublished observations, manuscript in preparation). Sporadic information on other polysaccharides such as arabinogalactans from larch [105, 106], grass pollen [107], salvia [108], and pectic arabinogalactans [109, 110] exists. However, as previously mentioned, differences in purity and methodology in assessment of immunomodulating properties, makes it difficult to get a clear picture of their potential. Further studies are required to fully understand the structure-function relationship in β-glucans and as well as in other polysaccharides.

In order to study and compare a broader range of different NSPs as regards their capacity to regulate immune responses in DCs, we recently performed a study testing 28 NSPs from different species and sources (unpublished data). The general outcome was that amongst the various different carbohydrate structures, only the mannan- and β-glucan-containing compounds comprised potent immunoregulatory activity, leading to down-regulation of TLR-induced IL-12p70 production, while enhancing TLR-induced IL-10. Besides TLR-induced modulation of cytokine production in DCs, all these bioactive NSP structures *per se* induced potent maturation of DCs leading to up-regulation of MHC class II and the co-stimulatory surface molecules CD40, CD80 and CD86, suggesting that they exert extensive modulation of the DC effector type. As β-glucans of both botanical and microbial origin were included, we could by direct comparison conclude that β-glucans of microbial origin are much stronger immunomodulators than plant-derived β-glucans, but we cannot exclude that a potent IL-10- and TNF-α inducing property amongst the microbial-derived β-glucans is due to the presence of immunostimulatory contaminants, such as lipoproteins present in e.g. the Zymosan, a particulate yeast preparation, or LPS determined to be present in a commonly used curdlan (linear β-(1,3)-glucopyranose) preparation.

IN VITRO EFFECTS OF NSP EXTRACTS ON MACROPHAGES AND DCS

Macrophages and DCs play a critical role in all phases of host defense including both innate and adaptive responses in case of infection. Basically, the immunoregulation induced by macrophages and dendritic cells is of identical nature, however, only DCs are capable of activating naïve $CD4^+$ T cells, and thus initiate activation of immune responses towards newly exposed antigens (i.e. peptides from phagocytosed intruders) [1]. Ligand binding by dectin-1 and other carbohydrate-binding PRRs on macrophages and DCs results in phagocytosis of the receptor-bound ligands (e.g.

intact yeast particles), and dose-dependent stimulation of nitric oxide (NO) and TNF-α production [111]. Enhancement of phagocytic activity seems thus to represent one important mechanism in the response to NSP.

Macrophages and NSP effects

Most studies investigating effects of polysaccharides from different natural sources on the response in macrophages have shown that NSPs derived from plants, algae and fungi basically all enhance macrophagal function (Table 2). The general functions are: Increased cytotoxic activity towards tumor cells, activation of phagocytosis, increased production of NO, and enhanced secretion of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6. Some report enhancement of IL-12p40, and an increase in apoptosis. While the involvement of the carbohydrate-binding receptors for enhancement of phagocytic activity in macrophages is evident [111], presently we can only assume that the described effects of the NSP extracts (Table 2) is due to NSP-induced activation of different carbohydrate-binding PRRs, as the receptors involved in the activity was not reported for these NSP extracts. Based on studies on pathogenic-derived ligands containing similar carbohydrate units (like mannosylated lipoarabinomannans (ManLAM) from Mycobacteria and β-glycans and mannans from yeast, the activation of phagocytotic activity in macrophages may be due to specific activation of the phagocytotic receptors such as dectin-1, MR and DC-SIGN (as displayed in Fig. 1). Specific activation of the carbohydrate-binding receptors by NSP extracts needs, however, to be verified in future studies.

Table 2: Effects of NSPs on macrophages and DCs.

NSP specie and source	Structure	Cell type	Effects	Ref.
Higher plants				
Leucaena leucocephala, chemically modified galactomannan	C-glycosidic 2-propanol derivates of (1,4)-β-D-mannopyranose backbone with (1,6)-α-D-galactopyranose side chains	Raw 264.7 murine MΦ	Inhibition of TNF-α and NO induced by LPS	[112]
Aloe vera var. *chinensis* *A*cemannan	(1,6)-α-D-mannoacetatepyranoside	Murine peritoneal MΦ	↑ NO, TNF-α, cytotoxity and phagocytosis	[100]
Oat, *Avena sativa*, β-glucan	(1,3),(1,4)-β-glucan	Murine peritoneal MΦ	↑ IL-1 and TNF-α	[113, 114]
Juniperus scopolorum, Arabinogalactan	(1,3)-D-galactopyranose with side chains of arabinosyl and galactosyl residues	Murine peritoneal MΦ Murine J744.1 MΦ	↑ NO, ROS, IL-1, IL-6, IL-12, TNF-α	[115]

Continued

Table 2: *Continued*

NSP specie and source	Structure	Cell type	Effects	Ref.
Tinospora cordifolia	(1,4)-α-glucan	Raw 264.7 murine MΦ	↑ TNF-α and NF-κB activity	[116]
Lycium barbarum	Man/Glu/Gal/Ara/Rha/Xyl	BMDC	↑ IL-12 p40	[117]
Algae				
Ulva rigida, Ulvan	Repeated disaccharide units of β-D-glucuronosyluronic acid (14) L-rhamnose-3-sulphate	Raw 264.7 murine MΦ	↑ NO and PGE_2	[118]
Fucus vesiculus, fucoidan	Sulfated (1,3)-fucoside	BMDC	↑ viability, IL-12, TNF-α, MHCI, MHCII, CD54 and CD86	[119]
Fungi				
Armillariella tabescens, α-glucan	(1,4),(1,6)-α-D-glucan	Murine peritoneal MΦ	↑ NO, IL-1β, IL-6, TNF-α	[120]
Saccharomyces cerevisiae, particulate β-glucan	(1,3),(1,6) -β-D-glucan	Murine peritoneal MΦ	↑ IL-6 and TNF-α	[121]
Cryptococcus neoformans, galactoxylomannan	α-(1,6)-galactan with side chains at C3 of α-D-mannan-(1,3)-α-D-mannan-(1,4)-β-D-galactan with terminal β-xylose	Raw 264.7 murine MΦ	↑ TGF-β, TNF-α and NO	[122]
Cryptococcus neoformans, glucuroxylomannan	(1,3)-α-D-mannopyranan bearing β-D-xylopyranosyl, β-D-glucopyranosyluronic acid, and 6-O-acetyl substituents	Raw 264.7 murine MΦ	↑ TGF-β, TNF-α and NO	[122]
Grifola frondosa grifolan	(1,3),(1,6) -β-D-glucan	Raw 264.7 murine MΦ	↑ IL-1, IL-6 and TNF-α	[123-125]
Cetratia islandica, lichenan	(1,3),(1,4)-β–D-glucan	Monocyte-derived DCs	↑ IL-10 and IL-12 p40	[96]
Peltigera canina, galactomannan	(1,4)-β-D-mannopyranoside backbone with (1,6)-α-D-galactopyranose side chains	Monocyte-derived DCs	↑ IL-10 and IL-12 p40	[96]
Thamnolia vermicularis var. *subliformis* Thamnolan	Gal/Rha/Glc/Xyl/Man	Monocyte-derived DCs	↑ IL-10 and IL-12 p40	[96]
Ganoderma lucidum β-glucan	(1,3),(1,6)-β-D-glucan	Monocyte-derived DCs	↑ IL-10 and IL-12 p70	[126]
Saccharomyces cerevisiae, zymosan	(1,3),(1,6) -β-D-glucan + (1,6)-α-D-mannan with α-(1,2) linked side chains	Monocyte-derived DCs	↑ IL-23 and IL-10	[127]

BMDC: Bone marrow derived DCs, MΦ: Macrophages.

NSPs and immune modulation in DCs

During the last decade the involvement of dectin-1 in induction of immune responses to β-glucan structures has been thoroughly examined. It is now evidently clear that the major cytokine induced by β-glucans in a dectin-1-dependent manner in DCs is the anti-inflammatory cytokine IL-10 [128, 129]. However, dectin-1 is synergizing with TLR2/TLR6, and presumably also other TLRs (own unpublished results), in production of IL-10 [128, 129]. Various studies have also shown that IL-10 is the main cytokine being produced after activation of MR and DC-SIGN in DCs [83, 85]. Most studies examining the effects of NSP extracts on DCs report the induction of IL-10, as well as IL-12, IL-23, or TNF-α (Table 2). Furthermore, increased surface marker expression of MHCs and co-stimulatory molecules (CD40, CD80, CD86, and CD54) is described. It has, however, not been examined which receptors that are involved in the reported effects. The described molecular modulation in DCs after NSP stimulation may be induced by various PRRs, including TLRs and NODs, besides the carbohydrate-binding PRRs. Although the potential for immune modulation by NSPs is reliable, presently, more studies are needed in order to elucidate the exact mechanisms of action of NSP extracts, and to characterize the bioactive structures within these preparations. Additionally, it is of importance to clarify whether other non-glycan components presents in the extract preparation could be responsible for the reported effects.

Immunostimulatory contaminants in NSP preparations

When testing the immunological effects of specific NSP structures, the possible co-presence of immunomodulatory contaminants is not negligible. The different NSPs that have been tested in a range of studies (Table 2) are often extracts from different sources and not 100 % pure. Therefore there is a risk of ascribing certain effects to a certain NSP structure, while it actually is caused by impurities.

Amongst highly stimulatory components that might be present in NSP preparations is the Gram negative-derived LPS being potent even in minute amounts and often found to be present in biological preparations. In addition to LPS, bacterial lipoproteins present in both Gram-positive and Gram-negative bacteria also have potent immune activating abilities. The presence of immunostimulatory LPS or lipoproteins in plant extracts of NSP was recently confirmed in a study by Pugh *et al*. [130] As they treated the samples with LPS-binding polymyxin B or lipoprotein lipase, the capacity of these NSP samples to stimulate NF-κB activation, leading to transcription of

several cytokine genes including IL-12, TNF-α and IL-10 in macrophages was eliminated. Various immunomodulatory effects also have been ascribed to fungal-derived β-glucans which is actually a matter of co-contamination of the β-glucan preparations with immunostimulatory products from fungi. One example is the use of zymosan that previously was extensively used to study β-glucan-mediated effects in DCs and macrophages. It is now evident that β-glucans are recognized solely by dectin-1, and not by TLR2/6 [129], as has previously been suggested. Rather a more complex signaling system is taking place, where simultaneous binding of agonists to dectin-1 and TLR2/6 (presumably lipoproteins from yeast cell walls) induces collaborative coupling of signaling cascades in DCs resulting in synergistically enhanced levels of IL-2, IL-10 and TNF-α [131]. Another example is the proposed induction of IL-12p40 production in DCs after zymosan stimulation. Although IL-12p40 production is induced in DCs after stimulation with zymosan, the IL-12p40 increase was largely due to a TLR2/TLR6-MyD88-dependent signaling pathway, and not to dectin-1-Syk pathways [129], thus suggesting that IL-12 production is due to presence of a TLR2 agonist in the zymosan preparation. Additionally, some of the effects attributed to curdlan, a (1,3),(1,6)-β-D-glucan-containing preparation derived from the Gram-negative bacteria *Alcaligenes faecalis* could well be due to presence of immunostimulatory LPS found to be present in significant amounts in a commercial preparation of curdlan (own unpublished results). As extraction of polysaccharides from fungi and higher plants rarely result in absolute pure monostructural components, impurities of other NSPs than the main polysaccharide, or the presence of potent immunostimulatory components, may explain the immunoregulatory or -stimulatory capacity.

ABSORPTION OF NSPS

NSPs are generally considered as components that are not digested and absorbed in the upper gastrointestinal tract of monogastric animals. Even though a large number of reports indicate that orally administered NSPs have different biological effects, only a few have considered the exact mechanisms of action *in vivo*. A few studies have reported absorption and pharmacokinetics of orally administered non-starch polysaccharides, but presently, very little is known about gastrointestinal absorption of NSPs.

Mostly the methods to detect the presence of polysaccharides in tissue and blood have involved isotope- or fluorescence labeling of the polysaccharide [132-136], or detection by the use of specific antibodies [136-139]. Covalent attachment of labels to the polysaccharide has the drawback of

chemical modification of the polysaccharide of interest, which may interfere with the natural mechanism of uptake [140].

Vetvicka *et al*. [135] illustrated in suckling rats that after a single dose of ^{125}I-phycarine, a (1,3)-β-d-glucan from the brown algae *L. digitata*, 25-29 % was detected in ileum after 30 min, 0.5-1.5 % were detected in the liver, 0.2-1.0 % in the kidney, and less than 0.5 % in the blood. This corresponds to the 0.5-5 % bioavailability estimated by Rice *et al.* [134] in rats fed different types of fluorescence-labeled water-soluble (1,3),(1,6)-β-D-glucans, indicating that only a very small part of orally administered β-glucan is absorbed from the gastrointestinal tract to the blood. However, Rice *et al*.[134] also showed that the bioavailability and plasma concentrations may be changed by structural conformation and charge [134], and discussed that insoluble particulate β-glucan may be absent from plasma, as they are phagocytozed and transported by macrophages as previously shown by Hong *et al*.[132].

Rice *et al*.[134] demonstrated that a subpopulation of the intestinal epithelial cells was able to actively absorb and internalize fluorescently-labeled β-glucan phosphate and, in addition, they also found glucan in cells isolated from Peyer´s patches after oral administration. NSPs may exert their action by getting in contact with the lymphoid tissue of the gut without emerging into blood. Microfold (M)-cells, located on top of the Peyer´s patches along the lining of intestinal epithelial cells, might be involved in uptake of high molecular weight glucans, as also suggested by Hashimoto and colleagues [141]. Proteins of molecular weight comparable to NSP have been shown to be absorbed by non-specific absorption mechanisms. By feeding mice *Tricholoma matsutake* (CM6271), Hoshi *et al*.[137] showed that a glycoprotein with a α-d-glucan sugar chain could be located in M-cells and later as well in Peyer´s patches. Not only glucans have been located in the Peyer´s patches after oral administration; bupleuran (a rhamnogalacturan) was detected in the T cell area of follicles in Peyer´s patches [138]. While these reports collectively point towards M-cells being capable of taking in NSPs, still, clear evidence for absorption of glucans into systemic circulation is lacking, as none of the previous studies did address whether the detected label was detached from the glucan after *in vivo* administration. Sandvik *et al.*[142] also disputed this and proposed that mucosal DCs are responsible for the uptake of polysaccharides. It is now well recognized that DCs sample or interact with gut contents locally via cellular projections that cross the epithelium [143]. Upon sampling they then migrate via afferent lymphatics to the mesenteric

lymph nodes, where immunomodulation is initiated [144]. Besides DC-mediated uptake of NSPs, it has been suggested that intestinal macrophages can take up β-glucans and transport them to the lymph nodes, spleen and bone marrow [145].

Hence, the uptake but also elimination rate/clearance of polysaccharides may be quite complex. Factors such as molecular charge, molecular size, branching frequency, solution conformation and degree of polymerization are important. This was clearly demonstrated in a study using intravenous (iv) administration of β-glucans, where glucan phosphate, laminarin and sclero-glucan were found to possess different pharmacokinetics [133].

Whether absorbed polysaccharides are distributed to systemic blood through the portal vein or the lymph is not completely clear. Rice *et al*. [134] concluded based on the finding of a 15 % lower concentration of β-glucan in plasma from vena cava compared to the hepatic vein that hepatic uptake did not contribute significantly to systemic clearance of β-glucans. Certainly, these results show that the liver is not very efficient in extraction of the β-glucans, but actually the results also point toward a net-absorption, which may suggest that hepatic uptake of β-glucan from the intestine could be a route by which β-glucans could enter systemic circulation.

Based on the common knowledge on uptake of food components, it is likely that the principal mechanisms for uptake of non-digestible glucans from the diet is due to absorption via M-cells as well as DC-mediated transfer and endocytosis, yet these issues still need to be fully verified in order to clarify the amounts and routes of transfer of NSPs to systemic circulation.

EFFECTS OF NSPS IN ANIMAL MODELS

A vast number of papers describing the *in vivo* biological effects of polysaccharides exist. A wide variety of animal species have been used in these studies: Fish, rats, mice, cattle, pigs, shellfish, dogs, chickens, rabbits as well as humans. The majority of the studies have been conducted in rats, pigs, and mice, and these will be the main focus here.

Anti-infectious activities of NSPs

An array of polysaccharides has been reported to influence pathogenic infections. In general, these studies show an increase in the clearance of pathogens and a reduced mortality of the infected animal (Table 3). *Staphylococcus aureus*-challenged rats that received yeast β-glucan injected intramuscularly showed a decrease in bacterial load [146]. In another study, mice infected with anthrax increased their survival rate after subcutaneous injection of yeast β-glucan [147]. Whether these effects take place due to immunomodulatory actions of the polysaccharide is not established, but the fact that the administration site of the polysaccharide was distinct from the challenge site supports this possibility. In contrast, another study reported increased mortality in pigs infected with *Streptococcus suis* when fed a β-glucan-containing diet [148].

The higher survival rate of infected animals after NSP administration is not well understood presently, but it might be due to a less violent immune response towards the bacteria, hence avoiding septic shock. This explanation is supported by the findings by Ahn *et al.*[149], showing that ginsan, consisting of β-(2,6)-fructofuranose and α-(1,6)-glucopyranose units modulated the inflammatory response in *S. aureus*-infected mice by down-regulating the inflammatory cytokines such as TNF-α, IL-1β, IL-6, IFN-γ, IL-12, and IL-18, while enhancing the production of the anti-inflammatory cytokine IL-10 in sera. The *in vivo* dampening of an inflammatory response by β-glucans, has also been shown in a sepsis model in rats [150].

The mechanistic justification for this effect is supported from *in vitro* experiments performed in our group, where we also have seen a downregulation of the proinflammatory cytokine IL-12p70 and no or an enhancement of IL-10 upon simultaneous stimulation of DCs with LPS and various NSPs (unpublished data).

Studies looking into effects of oat β-glucan in relation to improvement of an infection with the parasite *Eimeria vermiformis* in immunocompromised mice, suggested that oat β-glucan may partially restore the suppressed immune functions by enhancement of IgG immunity [151, 152].

Table 3: *In vivo* effects of various polysaccharides on microbial infections.

NSP specie	Observation	Ref
Yeast β-glucan	Increased survival rate of mice in a anthrax infection model	[147]
Yeast β-glucan	Lowering of *Staphylococcus aureus* level in infected rats	[146]
Yeast β-glucan	Increased disease susceptibility after *Streptococcus suis* challenge in pigs	[148]
Glucan Phosphate	Increased long-term survival of mice after infection with *Staphylococcus aureus* and *Canidia albicans*	[134]
Ginsan, a polysaccharide from Panax ginseng	Antiseptic mechanism induced by ginsan in mice infected by *Staphylococcus aureus*. Downregulation of inflammatory cytokines, such as tumor necrosis factor-α, interleukin (IL)-1β, IL-6, IL-12, IL-18 and interferon-γ	[149]
Polysaccharides extract from *Agaricus blazei* Murill	Anti-infection effects in mice towards *Streptococcus pneumoniae* serotype 6B.	[153]
Oat β-glucan	Enhanced survival of mice challenged with *Staphylococcus aureus*. Intraperitoneal administration of the glucan resulted in the accumulation of leucocytes, predominantly macrophages, in the peritoneal cavity	[113]
β-glucan from *Sclerotinia Sclerotiorum*	Curative effect on infection with *Streptococcus pneumoniae* serotypes 6B in mice	[154]
Oat β-glucan	Increased resistance to *Eimeria vermiformis* parasitic infection in immunosuppressed mice. Increase in IgG, IgG1, IgG2a, IgM and IgA immunoglobulins in the serum.	[151, 152]

Anti-tumor effects of NSPs

The polysaccharides have not only shown anti-inflammatory effects in *in vivo* studies, also anticancer effects have been reported (Table 4). Application of polysaccharides in anticancer treatment together with antitumor monoclonal antibodies (mAb) is a novel and promising treatment. Generally, the principal basis behind the effect of NSPs in regard to immunotherapy in cancer treatment is formation of opsonized iC3b (inactive product of cleaved complement fragment C3b) on the surface of tumor cells. The complement receptor 3, which is expressed on neutrophils, monocytes, NK cells and to a minor extend on macrophages and DCs [77, 155], has two separate binding sites: one for iC3b and another for β-glucans. When simultaneous ligation of both iC3b and β-glucan takes place, CR3 mediates cellular cytotoxicity towards iC3b-opsonized tumors [156]. The effectiveness in tumor regression capability of an existing antitumor mAb, Rituxan, was significantly increased when given in combination with oral β-glucan [157], thus demonstrating this mechanism in vivo.

Side effects of NSPs

Even though major pharmacological effects of NSPs are considered as safe, some unfavorable negative effects may be associated to these polysaccharides. High oral intakes of purified NSPs may induce gastrointestinal discomfort like bloating, flatulence, and liquid stool. Negative effects of the immune regulatory aspects should also be considered. Used as adjuvant, β-glucans from Candida albicans has been shown to trigger experimentally collagen-induced rheumatoid arthritis [158]. Injection of different β-D-(1,3)-glucans in combination with oral intake of a nonsteroidal anti-inflammatory drug (NSAID) can also induce lethal toxicity, where the most potent response modifiers are also giving rise the most exaggerated lethality when co-administered with the NSAID indomethacin [159]. Certain NSPs induce nitric oxide (NO) that has a cytotoxic effect on tumor cells and also an impact on many pathogens, but can at the same time also damage healthy tissue and DNA [160].

Table 4: Antitumor effects of different polysaccharides.

NSP specie	Observation	Ref
Yeast β-glucan	Together with mAb a tumor regression was reported in mice.	[132]
Alignates from *Sargassum Vulgare*	Inhibition of growth of sarcoma 180 tumor.	[161]
β-glucan from *Saccharomyces cerevisiae*	Stimulation of proliferation and activation of peripheral blood monocytes in patients with advanced breast cancer.	[162]
Polysaccharide–protein complex from *Lycium barbarum*	Antitumor effect on sarcoma 180 in mice. An increased IL-2 level and an increased phagocyticity in MΦ.	[163]
κ-Carrageenan	A dose dependent antitumor effect on sarcoma 180 in mice. The higher the degree of sulfonation, the higher tumor inhibition effect achieved.	[164]
β-glucan from *Aureobasidium pullulans* 1A1	Inhibition of tumor growth and liver metastasis in mice intrasplenically implanted with colon 26 cells and an increase in NK- and IFN-γ-positive cell numbers.	[165]
Yeast β-glucan	Combined immunotherapy with tumor antibodies was therapeutically effective against NCI-H23 human non small-cell lung carcinomas in mice.	[166]
Yeast β-glucan	Inhibition of lung tumor metastasis in mice by colon 26-M3.1 carcionomas. Activation of macrophages and NK-cells and an induced secretion of IL-1β, IFN-γ and IL-12.	[167]
λ-Carrageenan	Size dependent inhibition of growth of both S180 and H22 tumors in mice, where low MW products promote the highest antitumor activity.	[168]
SZP, a β-glucan-containing NSP	Mice with Ptas64 mammary tumors showed enhanced tumor regression when treated with SZP in combination with a mAb	[169]
Barley β-glucan, oat β-glucan and lichenan	Enhanced anti-tumor effect of mAb against established tumors in mice, irrespective of the route of β-glucan administration (intragastric or intraperitoneal), antigen (GD2, GD3, CD20, EGFR, HER2), human tumor type (NB, melanoma, epidermoid carcinoma, lymphoma, breast cancer), mouse strain (athymic nu/nu, severe combined immune deficiency mice), or tumor site (s.c. versus systemic)	[170, 171]

CONCLUSION

Although fibers of both microbial, fungal and plant origin have been demonstrated to hold immunomodulatory capacities both *in vitro* and *in vivo*, the potential of NSPs from microorganisms generally far exceeds that of plant NSPs. Certain chemical structures seem to hold particularly strong immunomodulating activities, including branched β-(1,3)-D-/β-(1,6)-glucopyranosyl polymers and derivates of mannans, but also physic-chemical parameters such as size and solubility are important for the activity. The conditions for in depth assessment of the most important factors and mechanisms involved in the biological activity are hampered by low purity of some of the studied fiber preparations, increasing the risk of presence of contaminating compounds with immunomodulatory activity, as well as by the lack of analytical techniques suitable for measurement of low NSP concentrations in body fluids and tissues. Still, although the mechanisms behind are far from understood, the effects on various disease animal models point towards great biomedical potentials of a number of polysaccharide structures.

THIS THESIS

This section introduces the objectives of the experimental studies presented in the chapter 3, 4 and 5.

In chapter 3 we screen a large panel of different non-starch polysaccharides in a murine bone marrow derived dendritic cell model. The aim of the study is to describe factors important for the immunoregulatory activities of the non-starch polysaccharides. The screening addresses the influence of chemical structure, size, origin and purity of non-starch polysaccharides for their capacity to interact with and regulate the phenotype of dendritic cells.

The aim of the study in chapter 4 is to study the regulation and induction of specific phenotypes of human monocyte-derived dendritic cells by a subgroup of the non-starch polysaccharides; β-glucan. We describe the regulation of TLR-triggered phenotype in human monocyte-derived dendritic cells by β-glucans. The designation of the phenotype would be based on an array of cytokines, chemokines and surface markers expressions levels.

Chapter 5 describes the *in vivo* modulatory properties of a β-glucan. We examine the β-glucan lichenan for its potential to engage as an adjuvant and to induce an antigen-specific response towards an antigen, with specific focus on the importance of the complex formation between protein and lichenan for its immunomodulating effect.

CHAPTER 3

COMPARATIVE ANALYSIS OF A LARGE PANEL OF NON-STARCH POLYSACCHARIDES REVEALS STRUCTURES WITH SELECTIVE REGULATORY PROPERTIES IN DENDRITIC CELLS

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Structural-based recognition of foreign molecules is essential for activation of dendritic cells (DC) that play a key role in regulation of gut mucosal immunity. Orally ingested non-starch polysaccharides (NSP) are ascribed many health-promoting properties, but currently we lack insight into the impact of structure and size for their capacity to affect immune responses. This paper addresses the importance of chemical structure, size, origin and presence of contaminants for the capacity of both dietary and non-food NSP to modulate DC.

Of 28 NSP products, β-glucans of microbial and plant origin and the galactomannan guar gum were found to modulate the DC cytokine pattern induced by the TLR4-ligand lipopolysaccharide (LPS) giving rise to reduced IL-12p70 and increased IL-10 levels, whereas IL-6 production was unaffected. A large proportion of the tested NSP were able to down-regulate LPS-induced IL-12p70 production. The most potent NSP induced up-regulation of CD86 on DC independently of LPS stimulation. Cereal-based β-glucans showed less potency than β-glucans of microbial origin, but proper molecular weight composition and preparation may improve effectiveness.

Collectively, this comparative study revealed that some plant-derived NSP besides those of microbial origin exert modulation of the DC phenotype, with the exact structure being important for the activity.

INTRODUCTION

Non-starch polysaccharides (NSP) are present in variable amounts in cell walls of plants and specific microorganisms, and several health-promoting properties are attributed the presence of certain NSP in food or in dietary supplements. It is widely accepted that many NSP hold prebiotic properties and improve gut transition time [172]. More recently, the attention on the direct immunoregulatory capacities of NSP has arisen; however, the knowledge regarding the immunoregulatory capacity of different NPS is limited. Improved understanding of the impact of specific molecular properties for the regulation of immune cells may facilitate implementation and marketing of their use as dietary supplements or support development of future therapeutic products.

Dendritic cells (DC) represent immune cells of importance for NSP regulatory properties. NSP recognition by DC is based on DC display of glycan-binding receptors, e.g. the C-type lectin receptors (CLR) [173]. CLR binding in DC has previously been reported to modify signals from other pattern recognition receptors (PRR), such as Toll-like receptors (TLR) [174], and NSP binding to other glycan-binding receptors may exert similar effects. DCs play a decisive role in orchestrating the immune response by acting as sentinels of foreign material and in activation of naive $CD4^+$ T helper (Th) cells, thereby bridging innate and adaptive immune responses [3]. Due to the central role of DC in $CD4^+$ T-cell polarization, modulation of DC activity by any compound will instruct adaptive immunity into an immunogenic (Th1, Th2, Th17) or tolerogenic (Treg) functional $CD4^+$ T-cell effector type [175, 176]. Depending on the type of DC modification, the effectiveness of both innate and adaptive immune responses may be altered. In terms of DC modulation, the production of IL-12p70 and IL-10, which are potent mediators for development of Th1 and Treg subsets, respectively, is affected [177, 178]. Moreover, the surface display of MHC class II and the co-stimulatory molecules CD80, CD86 and CD40 on DC is required to induce activation of $CD4^+$ T-cells [179]. NSP may interact with DC at the gut mucosal epithelial surface upon ingestion of NSP containing foods or dietary supplements. Sites for gut mucosal interaction between NSP and DC are at the M-cell interface upon NSP internalisation, or by direct paracellular uptake of NSP by DC that penetrate their dendrites through the gut epithelial tight junctions [137, 138, 143].

Various NSP from algae, fungi and higher plants have previously been shown to stimulate diverse components of the immune system, but with varying effect, in different experimental set-ups, and

presumably through different mechanisms. Most knowledge exists on the fungal β-glucans that are reported to interact with DC through the CLRs dectin-1 [180] and CR3 [78, 156]. β-glucans are polysaccharides containing glucose as the structural component, but with great variability in structural composition. Mixed-linked β-(1,3)(1,4)-D-glucans are abundant in cereals, mainly in barley and oat, while branched β-(1,3)(1,6)-D-glucans are found in fungi [181, 182]. β-glucans are also found as straight β-(1,3)-glucans (e.g. curdlan and paramylon) and β-(1,4)-glucans (cellulose) [183]. NSP comprised of other carbohydrates have attracted less attention than β-glucans, but may hold potent immunoregulatory properties as well. Not all NSP stimulate maturation of DC, but may nevertheless modulate the maturation of DC induced by a microbial signal, such as lipopolysaccharide (LPS).

In order to enhance our understanding of factors important for the immunoregulatory activities of these complex nondigestible polysaccharides, we here screened a range of different NSP addressing the influence of chemical structure, size, origin and purity of NSP for their capacity to interact with and regulate the phenotype of DC.

MATERIALS AND METHODS

NSP preparations

NSPs were obtained in purified form from different chemical companies, as experimental extract preparations, and in the form of commercially available food-grade preparations. Wheat arabinoxylan, xyloglucan from tamarind, arabinan from sugar beet, larch wood arabinogalactan, rhamno-galacturonan and galactan extracted from potato, konjac mannan from *Arnorphophallus konjac,* curdlan from *Alcaligenes faecalis,* lichenan from *Cetraria islandica,* pullulan from *Aureobasidium pullulans*, yeast β-glucan, high and medium viscosity oat β-glucan (OBG4, 5), and low, medium, and high viscosity barley β-glucan (BBG4-6) were purchased from Megazyme International Ltd., Wicklow, Ireland. Gum Arabic from Acacia, agar from Gracilaria seaweeds, xanthan from *Xanthomonas*, guar gum from *Cyamopsis tetragonolobus campestris*, locust bean gum from *Ceratonia siliqua*, ι- carrageenan from *Eucheuma spinosum*, κ-carrageenan from *Eucheuma cottonii*, λ- carrageenan from *Chondrus crispus*, Zymosan from *Saccharomyces cerevisiae*, Paramylon from *Euglena gracilis*, microcrystalline cellulose, and amylose from potato were purchased from Sigma-Aldrich Inc. St. Louis, MO.

Chicory inulin (Raftiline HP) was purchased from Orafti, Tienen, Belgium, sugar beet pectin from CP Kelco, Lille Skensved, Denmark, dextran (T2000) from *Leuconostoc mesenteroides* from Pharmacia Biotech Uppsala Sweden, while enzymatically modified barley β-glucan was provided by Crops & Food Research, Christchurch, New Zealand (BBG1-3). Isphagula (*Plantago ovata*) was bought in a local drugstore. PromOat (OBG1) was obtained from Biovelop, Höganäs, Sweden. Two barley β-glucans (BBG7, 8) were supplied by Novozymes, Bagsværd, Denmark.

Pilot plant scale preparations of oat β−glucan were kindly provided by Dr. Peter J. Wood, Agriculture Canada, Guelph, Canada (OBG2), and by Prof. (emer.) Yrjö Mälkki, Cerefi Ltd., FI-02160 Espoo (OBG3).

The enzymatically-modified barley β-glucans (BBG1-3) were prepared by the following procedure; barley flour was extracted in water (1:7.5) by stirring the mixture at 45ºC for 45 minutes. After centrifugation, the supernatant was heated to 85 - 90ºC for 30 minutes with occasional stirring. After a second centrifugation, the β-glucan extract was treated with cellulase (*endo*-1,4-β-D glucanase) from *Trichoderma Longibrachiatum* (Megazyme International Ltd., Wicklow, Ireland) at three different enzyme concentrations (0.6, 6 or 60 U/ml) at 55ºC for 60 minutes with constant stirring. Following the incubation, the samples were frozen overnight and thawed the following day. The formed gel was collected on a 45 μm mesh, gently washed, and freeze dried.

NSP analysis

Except for some of the β-glucan preparations all fiber sources were analyzed for their content of neutral and acidic polysaccharides (uronic acids) as described by Bach Knudsen [184].

Determination of molecular weight

Samples were dissolved in water containing 0.02 % sodium azide for 2 h at 70°C after pre-wetting with 50 % ethanol. The molecular weight was determined by gel-permeation chromatography (GPC) using a Water HPLC Module 1 (Waters Corporation, Milford, USA) fitted with a series of 3 columns; TSKgel GMPWxl (Tosoh Bioscience LLC, Montgomeryville, PA) Shodex B-806 HQ and SB-806M HQ (Showa Denka K.K., Tokyo, Japan) and a Water 2410 Refractive Index (RI) detector (Waters Corporation, Milford, USA) using 0.2 M acetate buffer containing 2 g oxalic acid/l as eluent at a flow rate of 0.5 ml/min. The samples were calibrated against pullulan (Shodex P-82) standards (5.8, 12.2, 23.7, 48.0, 100, 186, and 380 kDa measured by an ultra centrifugal

sedimentation equilibrium method by the manufacturer, Showa Denko K.K., Tokyo, Japan) and in the case of β-glucan also by (1,3),(1,4)-β-D-glucan standards (40, 82, 123, 183, and 245 kDa as measured in 50 mM sodium hydroxide on Hydrogel 2,000, 500, 200 columns at 70°C, and dual angle light scattering detector fitted inside WATERS M411 refractive index detector by the manufacturer, Megazyme International Ltd., Wicklow, Ireland). Molar mass values calculated relative to the β-glucan and pullulan standards using the Waters Millenium32 software are reported in weight average (Mw).

Generation and stimulation of bone marrow-derived DC

Bone marrow (BM) cells were isolated from C57BL/6 mice (Taconic Europe, Denmark) as described previously [185]. To cultivate DC, 10 mL cell suspension containing $3 \cdot 10^6$ stem cells was seeded in 100-mm bacteriological petri dishes at day 0 (Greiner bio-one, Kremsmünster, Austria) and incubated for 8 days at 37 ^{o}C and 5 % CO_2. On day 3, an additional 10 ml cell culture medium was added. At day 6, cell culture medium was replaced by fresh medium. On day 8, the non-adherent cells were gently pipetted from the Petri dishes and centrifuged for 5 min at 280 g. The cells were resuspended in fresh cell culture medium without GM-CSF, and seeded in 48-well culture plates (Corning inc., Corning, NY) at $1 \cdot 10^6$ cells/600 μl well. DCs were cultured with various NSP with or without LPS (*Escherichia coli* O26:B6; Sigma-Aldrich Inc.) in a final concentration of 1 μg/mL. Cells added medium alone were used as untreated DC. After stimulation for 18 h, culture supernatants were collected and stored at -20 ^{o}C until cytokine analysis.

All animals' studies were approved by The Danish Animal Experiments Inspectorate and were carried out according to the guidelines of "The Council of Europe Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific purpose". Permission number: 2007/561-1266.

Cytokine quantification in culture supernatants

IL-6, IL-10, IL-12p70 and TNF-α were analyzed using commercially available ELISA kits (R&D systems, Minneapolis, MN) according to the manufacturer's instruction. Detection limits: IL-6: 4 pg/mL, IL-10:8 pg/mL, IL-12p70: 10 pg/mL, TNF-α: 10 pg/mL.

Analysis of surface molecule expression on DC

DC were generated and stimulated as described above except for seeding in 12-well culture plates (Nunc, Roskilde, Denmark) at day 0. Upon stimulation at day 8, cells were treated with ice cold PBS-az; containing 1% (v/v) heat-inactivated FBS and 1.5% (w/v) sodium azide (Sigma-Aldrich Inc.) to prevent internalization of surface markers during subsequent handling of the cells. DCs were thereafter kept at 4 ^{o}C or below. To block non-specific binding of Abs, cells were incubated with anti-mouse FcγIII/II receptor antibody, clone 2.4G2 (3 μg/ml, BD Bioscience, San Jose, CA) before addition of fluorochrome-conjugated Ab. Upon incubation, cells were washed twice in PBS-az before analysis on a BD FACSCanto II (BD Bioscience). The analysis was based on 20,000 cells and gated on viable cells. The Abs used were: APC-conjugated anti-mouse CD11c, clone N418, PE-conjugated anti-mouse CD40, clone 1C10, PE-conjugated anti-mouse CD80, clone 16-10A1, PE-conjugated anti-mouse MHCII, clone NIMR-4, all eBioscience, San Diego, CA. APC-conjugated anti-mouse CD86, clone GL1 (Southern Biotech, Birmingham, AL). Isotype-matched controls: APC-conjugated Rat IgG2a, clone R35-95 (BD Bioscience), PE-conjugated rat IgG2b, clone KLH/G2b-1-2 (Southern Biotech), PE-conjugated Armenian hamster IgG, clone eBio299Arm (eBioscience), PE-conjugated Rat IgG2a (eBioscience).

Data were analyzed using FCS Express (version 3.0, De Novo Software, Los Angeles, CA).

DC purity was verified by flow cytometry using the DC marker CD11c together with microscopic inspection. The proportion of $CD11c^{+}$ cells was 80-90 %.

Endotoxin test

NSPs were dissolved in endotoxin-free water to a final conc. of 1 mg dry matter/mL, mixed for 1 hour by continuous shaking and afterwards centrifuged at 280 g for 10 min. Supernatants were collected and tested for endotoxin content using the commercially available test: Pyrochrome (Cape Cod inc., E. Falmouth, MA) according to the manufacturer's instruction. The *Limulus* amebocyte lysate was reconstituted in Glucashield (Cape Cod) to prevent false positive results due to the presence of β-glucan.

Statistics

Data were analyzed for statistical significance (GraphPad Prism, version 4.03, GraphPad Software, San Diego, CA) using one-way ANOVA and the Dunnett test (comparing individual treatments with that of a control treatment (LPS or medium)). A P-value < 0.05 was considered statistically significant.

RESULTS

The influence of NSP structure and origin on modulation of LPS-induced cytokine production

Twenty-eight NSPs originating from different plant and microbial sources were characterized in regard to their DC modulating potency (Table 1). The NSPs were selected to obtain polysaccharides both of microbial and plant origin in different structural categories of which some had previously been described as having potential immune regulatory activities as well as NSP not former reported to be immune regulatory but having structural familiarities. A high number of β-glucans were included with the aim of studying the effect main structure, origin and molecular size.

Table 1. Characteristics of fiber preparations.

	Chemical structure	Mw[a] (kDa)	Mw relative to pullulan (kDa)	Mw relative to β-glucan (kDa)	NSP (% dm)	Purity[a] (%)	Other supplier information[a]
Pectin	Galacturonan	n.a.	784	n.r.	84	n.a.	
Gum Arabic	Arabinogalactan	250	n.a.	n.r.	97	n.a.	
Inulin	1,2-β-D-fructan	3.6	4	n.r.	99.9*	n.a.	Average DP 25, distribution 11-60 without sugars or polymers < 11 DP.
Rhamno-Galacturonan	Rhamno-Galacturonan	n.a.	15	n.r.	47	> 97	Galacturonic acid 62 %, Rhamnose 20 %, Arabinose 3.3 %, Xylose 1 %, Galactose 12 %, other sugars <0.1 %
Agar	β-1,3-alternating 1,4 linked 3,6-galactan, methoxyl, sulphate ester or pyruvate acetal substituted	n.a.	315	n.r.	94		89 % dm, 3.1 % ash, 0.2% foreign organic matter, 0.2 % foreign insoluble matter, viscosity 18 cP
Arabinoxylan, wheat	1,4-β-D-xylose backbone with 1,2 or 1,3 linked L-arabinofuranosyl residues	n.a.	1600	n.r.	86	> 97	
Isphagula	1,4-β-D-xylose backbone with 1,2 linked L-Ara*f* and 1,3 linked L-Ara*f*-α-(1,3)-D-Xyl*p*-β-(1,3)-L-Ara*f*.	n.a.		n.r.	91		
Xanthan gum	1,4-β-D-Glcp backbone substituted with manno-glucuronic-mannosid units	n.a.	n.a.	n.r.	65	n.a.	
Xyloglucan	1,4-β-D-Glc*p* backbone substituted at O6 with mono-, di-, or triglycosyl units	202	3500	n.r.	94	> 97	Sugar composition: 35 % xylose, 45 % glucose, 16 % galactose, 4 % arabinose
Arabinan	1-5-α-L backbone 1-3-α-L linked arabino-furanosyl residues	n.a.	70	n.r.	88	~ 95	Sugar composition: 88 % arabinose, 3 % galactose, 2 % rhamnose, 7 % galacturonic acid

Continued

Table 1: *continued*

	Chemical structure	Mw[a] (kDa)	Mw relative to pullulan (kDa)	Mw relative to β-glucan (kDa)	NSP (% dm)	Purity[a] (%)	Other supplier information[a]
Arabinogalactan	1,4-β-D-galactan with β-D-Galp or α-L-Araf and dimer/trimer galactosid-/arabino-galactosid residues	n.a.	18	n.r.	100	> 95	Purified by ultrafiltration. Sugar composition: 81 % galactose, 14 % arabinose, 5 % other sugars
Galactan	1,4-β-D-galactan	n.a.	240	n.r.	80	n.a.	Sugar composition: 88 % galactose , 3 % Arabinose, 3% rhamnose, 6 % galacturonic acid.
Konjac mannan	β-1,4-D-glucose and D-mannose	n.a.	3900	n.r.	94	> 98	Protein (N x 5.7) < 0.2 %, sugar composition: 60 % mannose, 40 % glucose, galactose, arabinose and xylose undetectable.
Guar gum	1,4-β-D-mannan substituted with single 1,6-β-D-galactosid	n.a.	n.a.	n.r.	92		89.5 % dm, 0.65 % ash.
Locust bean gum	1,4-β-D-mannan substituted with single 1,6-β-D-galactosid	310	n.a.	n.r.	88		89 % dm, 0.4 % ash, viscosity 2700 cP
λ-carrageenan	β-1,4-α 1,3-D-galactan, sulfated	n.a.	3700	n.r.	64§	n.a.	
ι-carrageenan	β-1,4-α 1,3-D-galactan, sulfated	n.a.	4000	n.r.	54§	n.a.	
κ-carrageenan	β-1,4-α 1,3-D-galactan, sulfated	n.a.	2300	n.r.	58§		5.6 % K, 2.7 % Ca, 0.6 % Na
Pullulan	1,4-1,6-α-D-glucan	n.a.	300	n.r.	94	> 95	96 % dm, < 0.3 % protein, ~3 % ash. Viscosity 2-3 cP at 1 % 30 °C.
Dextran	a-D-1,6-glucan with 1,3-a-D-glucopyranosyl side chains	2000	n.a.	n.r.	99	n.a.	
Curdlan	1,3-β-D-glucan	n.a.	n.a.	n.a.	93	> 99	
Paramylon	1,3-β-D-glucan	~ 500	45	45	103	n.a.	
Zymosan	1,3-1,6-β-D-glucan	n.a.	n.a.	n.a.	83	n.a.	93 % dm, 1.9 % nitrogen, 0.8 % phosphorus
Yeast β-glucan	1,3-1,6-β-D-glucan	n	80	55	n.a.	> 90	97.8 % dm, < 0.1 % α-glucan , 0.1 % protein, 0.1 % ash.
Cellulose	1,4-β-D-glucan	< 50	n.a.	n.a.	110	n.a.	95-97 % dm, <0.05 %, <0.25 % water soluble substances, < 0.05 % ether soluble substances , DP<350
Lichenan	1,3-1,4-β-D-glucan	n.a.	170	94	96	> 85 (99.5 % NSP)	98 % glucose with 85 % as 1,3-1,4-β-D-glucan, the remainder possibly isolichenan (α-linked form), < 0.1 % starch, 1.5 % arabinose, undetectable mannose, xylose and uronic acids.
BBG1	1,3-1,4-β-D-glucan	13	25	35	76	n.a.	
BBG2	1,3-1,4-β-D-glucan	65	110	75	80	n.a.	
BBG3	1,3-1,4-β-D-glucan	125	215	110	81	n.a.	
BBG4	1,3-1,4-β-D-glucan	137	350	150	n.a.	n.a.	98 % dm, 1 % protein, <0.0 % starch. Viscosity 5 cSt at 1 % 30 °C
BBG5	1,3-1,4-β-D-glucan	260	620	215	n.a.	~ 96	97.8 % dm, 0.4 % protein, <0.3% arabinoxylan, <0.1 % starch, 0.3 % ash. Viscosity 28 cSt at 1 % 30 °C
BBG6	1,3-1,4-β-D-glucan	320	2300	430	n.a.	n.a.	96.4 % dm, 1.2 % protein, <0.1 % starch. Viscosity >100 cSt at 1 % 30 °C
BBG7	1,3-1,4-β-D-glucan	4.6	5	20	n.a.	n.a.	
BBG8	1,3-1,4-β-D-glucan	8.6	10	25	n.a.	n.a.	
OBG1	1,3-1,4-β-D-glucan	>1500	1300	320	n.a.		93.3 % dm, 34-36 % soluble β-glucan, 35-37 % total DF, 54-56 % maltodextrins, 2.5-3.5 % protein, 3-4 % ash , 0.5-1 % fat.
OBG2	1,3-1,4-β-D-glucan	1175	3200	485	n.a.	n.a.	81 % β-glucan, 2 % starch
OBG3	1,3-1,4-β-D-glucan	n.a.	75	60	n.a.	n.a.	
OBG4	1,3-1,4-β-D-glucan	n.a.	700	220	n.a.	> 97	95 % dm, < 0.1 % starch , < 0.5 % arabinoxylan , 0.35 % protein, 0.5 % ash. Viscosity 20-30 cSt at 1 %.
OBG5	1,3-1,4-β-D-glucan	n.a.	1000	290	n.a.	> 97	98.8 % dm, < 0.1 % starch , < 0.5 % arabinoxylan , 0.3 % protein, 1.7 % ash. Viscosity 69 cSt at 1 %.

[a]Information provided by supplier, n.a.: not available, n.r.: not relevant, DP: degree of polymerization, cP: centipoise, cSt: centistokes.
*determined by HPLC.
§without sulfate. λ-,ι-, and κ-carrageenan were analysed to contain 20, 30, and 26 % ash, respectively.

To examine how NSP modulation of TLR-primed DC affects levels of proteins of importance for immune regulation and especially CD4 T helper cell differentiation, we evaluated the secretion of IL-12p70 (Th1 polarization) and IL-10 (Treg and/or Th2), and the levels of expression of MHC class II important for antigen presentation, as well as the co-stimulatory surface molecules CD80 (Th1), CD86 (not Th1) and CD40 (Th1) in DC. Moreover, we determined the levels of the general pro-inflammatory cytokine TNF-α, and the pleiotropic cytokine IL-6, which is involved in Th17 generation, and is generally up-regulated during pro-inflammatory conditions *in vivo*. To study how NSP modulate TLR-primed DC, the TLR4-ligand LPS was added to DC simultaneously with the individual NSP. TLR-triggering is needed as we have noticed that IL-10 and IL-12p70 is not measurable in DC upon incubation with TLR-free CLR-ligands. NSP from various sources can contain a variety of different TLR-ligands in varying amounts giving rise to variable effects in DC, but due to the nature of NSP it is not possible to purify for unknown microbial TLR-ligand contaminants. Addition of a high amount of LPS to all stimuli will level out these differences.

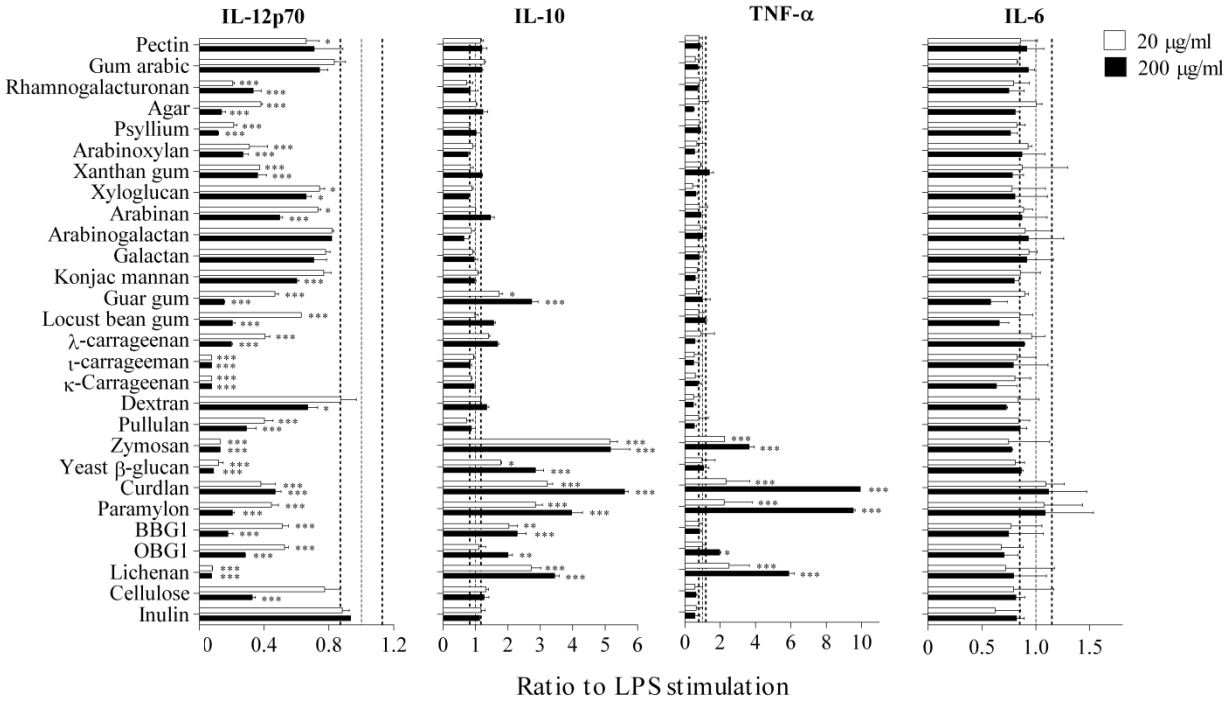

Figure 1. **Influence of NSPs on LPS-induced cytokine production in DC.**
Levels of cytokines in culture supernatants from murine BM-derived DC upon culturing for 18 h with indicated NSP at 20 or 200 μg/ml in the presence of LPS (1 μg/mL) determined by ELISA. Data are presented as the ratio to the LPS stimulation alone (mean ± SD, n=2). The solid line represents cytokine production from LPS-treated DC and the dotted line the SD from these cells. Absolute values in LPS-treated DC were as follows: IL-6: 80 ± 8 ng/mL, IL-10: 945 ± 150 pg/mL, IL-12p70: 570 ± 33 pg/mL and TNF-α: 21 ± 2 ng/mL. All cytokine concentrations in DC cultured with medium alone were below detection limit. Differences between dual-treated DC (NSP plus LPS) as compared to DC treated with LPS alone were tested by one-way ANOVA and the Dunnett test. $P < 0.05$, *, $P < 0.01$, ** and $P < 0.001$, ***. Data are representative of three experiments.

The NSPs differentially affected the LPS-induced production of the cytokines IL-12p70, IL-10, TNF-α and IL-6 in DC (Fig. 1). A diverse group of NSP was capable to suppress LPS-induced IL-12p70 production. Among those, the group comprising differently structured β-glucans and the galactomannan guar gum was at the same time able to augment the LPS-induced IL-10 production by a factor of 2 to 5. The microbial-derived β-glucans curdlan and zymosan showed the highest capacity to enhance LPS-induced IL-10 production, while lichenan, paramylon, yeast β-glucan, the cereal-derived β-glucans from barley and oat, and guar gum had a less pronounced but significant and dose-dependent effect. The 1,3-1,4-β-glucan lichenan and the β-glucans of microbial origin (curdlan, paramylon and zymosan) also enhanced LPS-induced TNF-α production by a factor of 4 to 9, with curdlan and paramylon being most potent. No significant effects of any of the tested NSP were observed in regard to modulation of LPS-induced IL-6. Importantly, the NSP inducing the highest level of IL-10 and TNF-α did not give rise to the strongest IL-12p70 inhibition. This is of importance as it has earlier been reported that enhanced levels of IL-10 inhibit IL-12p70 production [186]. The present data therefore indicate that the β-glucans and the galactomannan guar gum induce a specific regulatory pattern in DC leading to a synergistically increased IL-10 and TNF-α production and suppression of IL-12p70 upon LPS-stimulation.

Table 2. Endotoxin content in selected fibers

Fiber	LPS (EU/mg fiber)
Guar gum	< 0.6
Zymosan	< 6
Yeast β-glucan	0.7
Curdlan	100
Paramylon	1
OBG1	< 0.6
OBG4	< 0.6
BBG4	18
BBG8	0.8
Lichenan	3

Effects of NSP per se on cytokine production in DC

To evaluate the effect of NSP on DC without simultaneous presence of LPS, all NSP showing significant modulatory properties on LPS-induced IL-10 production in DC were examined for their capacity to stimulate DC *per se*. The production of IL-12p70, IL-10, TNF-α and IL-6 by DC upon exposure to 200 μg/ml of NSP was measured and compared with the concentration of cytokines

produced by LPS-stimulated DC (Fig. 2A, B). Only curdlan gave rise to detectable amounts of IL-12p70 (Fig. 2B) with levels comparable to those obtained with LPS alone (Fig. 2A). The microbial-derived β-glucans, zymosan and curdlan were the only NSP that induced significant levels of IL-10 in DC *per se*. Lichenan, curdlan and paramylon gave rise to TNF-α induction similar to that induced by the NSP when co-administered with LPS, while most NSP gave rise to some IL-6 production by themselves. Only zymosan and curdlan induced IL-6 comparable to the levels induced by LPS alone and did not exhibit additive effects with LPS. As the preparations, due to the microbial origin, and the manufacturing processes, may contain impurities with immunostimulating capacity, the endotoxin levels in the most potent preparations (Table 2) were tested. Amongst the products tested, the curdlan preparation contained the highest LPS content (100 endotoxin units (EU) per mg). Based on the approximation that 1 EU equals 0.2 ng LPS [187], we estimated the LPS contamination to correspond to administration of approx. 4 ng/mL LPS in curdlan stimulation of DC cultures, thus representing a minor, but stimulatory dose.

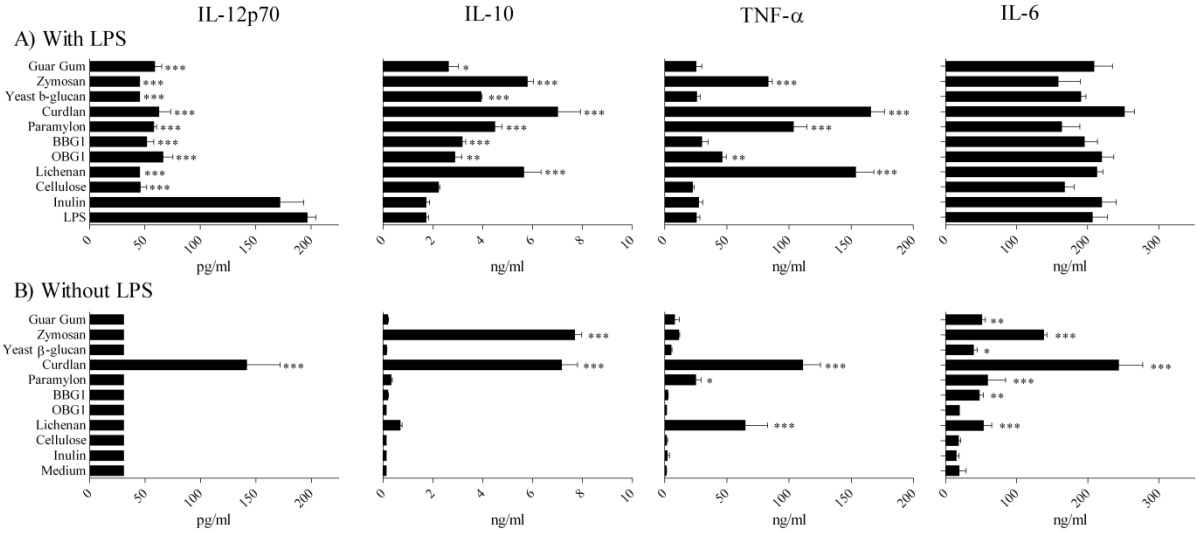

Figure 2. Cytokine production induced in DC by NSP *per se*.
Murine BM-derived DCs were cultured for 18 h with NSP at 200 μg/mL with 1 μg/mL LPS (A) or without (B), and cytokines present in the culture supernatants were measured by ELISA. Data represent the mean ± SD (n=4). Data were tested for statistical significance by one-way ANOVA and the Dunnett test. $P < 0.05$, *, $P < 0.01$, ** and $P < 0.001$, ***. Data are representative of three experiments.

Up-regulation of surface molecule display on DC by specific NSP

The NSP were also tested for their ability to induce or modulate the expression of the surface molecules MHC class II, CD40, CD80 and CD86 in DC. DC were cultured with NSP (200 μg/ml) either alone or together with LPS (Fig. 3). The NSP showing most pronounced effects on LPS

induced cytokine production in DC (increase of IL-10 and decrease of IL-12) significantly enhanced CD86 display on DC *per se* (Fig. 3A, B). Only lichenan reduced CD80 surface expression significantly as compared to medium-treated DC (Fig 3A, B). MHCII and CD40 were not affected by any NSP *per se* (Fig 3A, B).

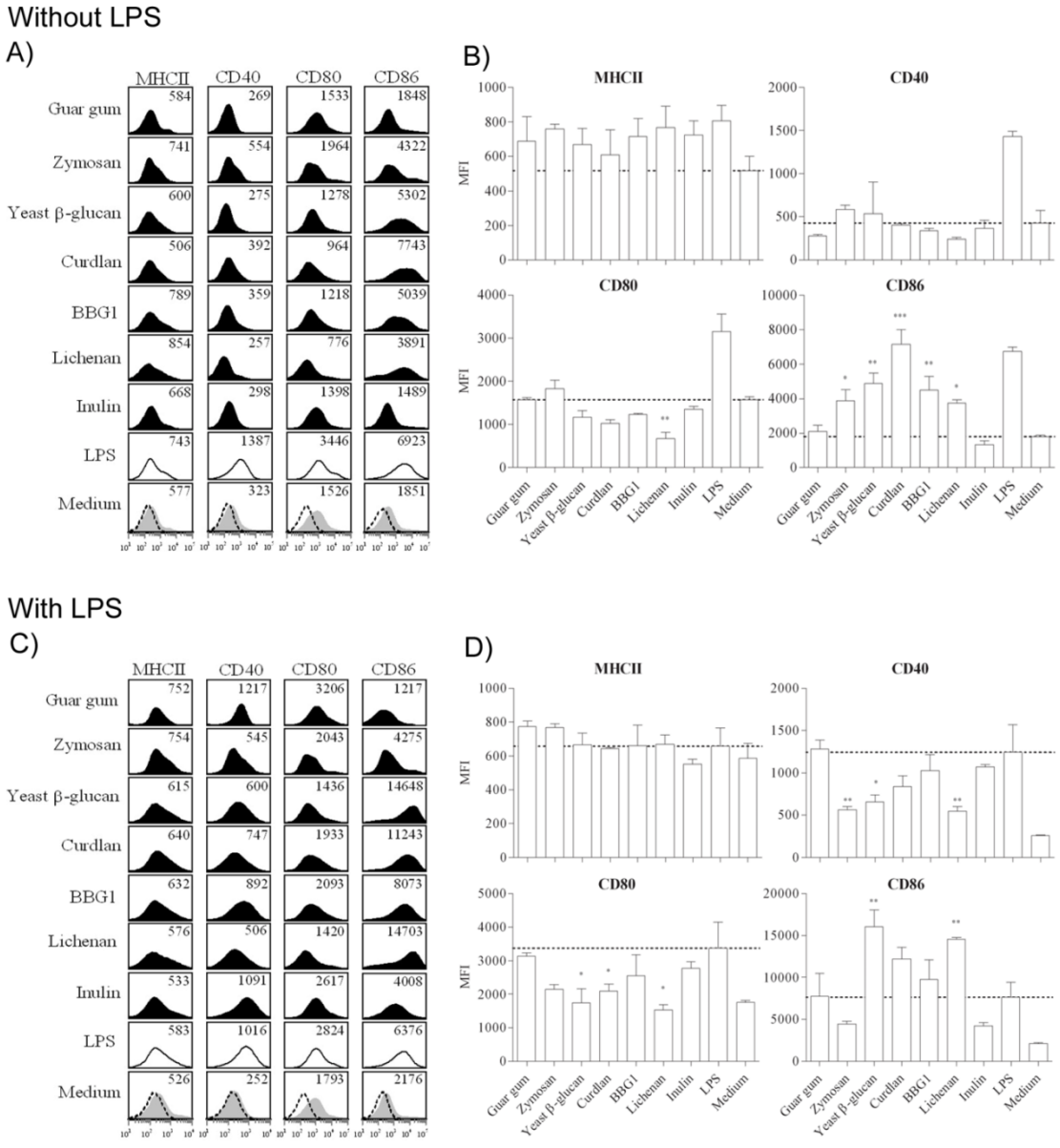

Figure 3. Modulation of MHC class II, CD40, CD80 and CD86 surface display on DC.
Murine BM-derived DC were cultured for 18 h with NSP at 200 μg/mL without (A, B) or with (C, D) addition of LPS at 1 μg/mL. Surface phenotype of DC was determined by flow cytometry after staining cells with PE-MHC class II (MHCII), PE-CD40, PE-CD80 or APC-CD86 mAbs. A, C: Histograms of surface expression levels of MHCII, CD40, CD80 and CD86. Isotype controls for each antibody-conjugate are shown by the dashed line in the medium-treated DC diagrams. B, D: The mean fluorescence intensity (MFI) of the surface markers. Data represent mean + SD from two independent experiments. Differences between treatments and medium-treated DC (A, B) or LPS-treated DC (C, D) were analyzed by one-way ANOVA and Dunnett test. $P < 0.05$, *, $P < 0.01$, ** and $P < 0.001$, ***.

As regards modulation of LPS-induced maturation of DC, yeast β-glucan and lichenan enhanced the CD86 expression, while they reduced the expression of CD40 and CD80. Curdlan reduced the expression of LPS-induced CD80, while zymosan reduced CD40 display on DC. Collectively, all β-glucans showed capacity to enhance surface expression of CD86 in immature DC by at least two-fold, but in the presence of LPS, we found more diverse modulatory properties amongst the β-glucans, and observed a reduction of the CD80 and CD40 surface display by several of these compounds.

Effect of source and molecular weight of cereal β-glucans on modulatory capacity in DC

In our initial screenings of the different NSP preparations, the cereal β-glucans showed some regulatory potential, although their effects were clearly less potent than another β-(1,3),(1,4)-glucan; lichenan. To further identify factors of importance for the activity, we compared immunomodulatory activity of a number of cereal β-glucans to their Mw and supplier information (Table 1). Mw of all products to be compared were determined under identical analytical conditions by size exclusion chromatography against β-glucan as well as pullulan standards (Table 1).

The cereal β-glucan preparations exhibited great variability in their capacity to modulate LPS-induced cytokine production in DC. Five barley β-glucans (BBG1, 3, 4, 5, 8) and one oat β-glucan (OBG1) were able to enhance the IL-10 production concurrent with suppression of IL-12p70 (Fig. 4). The active BBG preparations were obtained from all three suppliers, and showed a size dependency, as only samples between 25 and 215 kDa exhibited immunoregulatory effect, while samples of higher or lower molecular weight did not. The TNF-α production in LPS-activated DC was enhanced by three of these products (BBG4, 5, 8), while the barley β-glucan products (BBG4, 5) from one provider also induced significant amounts of IL-6 (Fig. 4A). In order to assess a possible role of contaminating LPS in these preparations, the endotoxin content in BBG1 and BBG4 was analyzed (Table 2). We detected LPS in both products, and although the levels were below 20 EU/mg it cannot be excluded that this amount of LPS is capable of modulating especially TNF-α induction in DC.

For the different oat β-glucan preparations, we observed an IL-10 and IL-12p70 modulatory effect of OBG1 only (Fig. 4B). This effect is not seen in the other product of similar size (OBG5), or in lower or higher Mw products (OBG2, 3 or 4). Accordingly, it was not possible to relate the activity

of the OBG products to the Mw. The endotoxin content in OBG1 and OBG4 was determined, and found to be <0.6 EU/mg, and thus unlikely to induce significant production of TNF-α and IL-6.

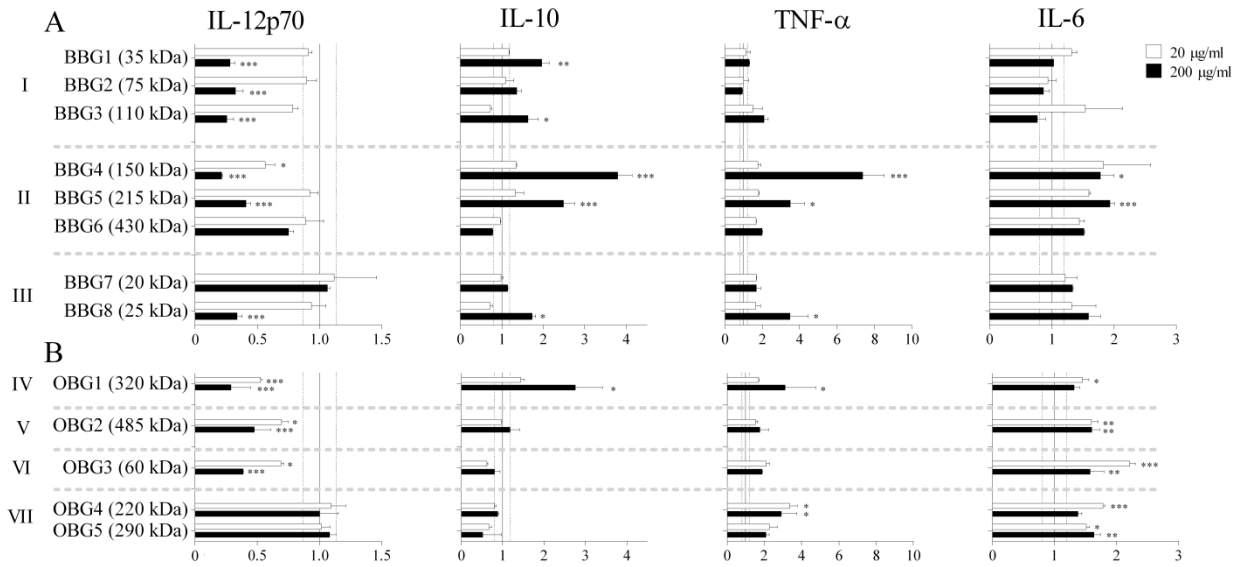

Figure 4. Effect of different molecular sizes and supplier of cereal β-glucans on cytokine-inducing potency in DC. Cereal β-glucans were obtained from different suppliers, as indicated by the roman numerals (I: GraceLinc, II and VII: Megazyme, III: Novozymes, IV: Biovelop, V: Agriculture Canada, VI: Cerefi), and molecular sizes were determined by size-exclusion chromatography (Table 1). The immunomodulating property of these cereal β-glucans was measured by culturing of BM-derived DC for 18h with 1 μg/mL LPS together with the different barley β-glucans (BBG) (A) or oat β-glucans (OBG) (B) at 20 or 200 μg/mL. Data are presented as the ratio to the LPS stimulation alone (mean ± SD, n=2). The solid vertical line represents the cytokine production in LPS-treated DC and the dotted vertical line indicates ± SD. Differences between treatments were analyzed by one-way ANOVA and Dunnett test (as compared to LPS-treated DC). $P < 0.05$, *, $P < 0.01$, ** and $P < 0.001$, ***. Data are representative of two experiments.

DISCUSSION

The central goal of our study was to address the structural and molecular basis for immunoregulatory capacity in DC amongst NSP that are part of our common foods or may hold potential uses as food supplements. We focused our evaluation on a broad panel of NSP with different structure, size and origin, in order to identify potent immunoregulatory molecules that may be able to modulate the functional phenotype of DC.

Our present work demonstrated the existence of a large variability in terms of DC immunoregulatory properties amongst various NSP structures. We found that greatest DC modulatory potency lies within the group of microbial-derived β-glucans, with the cereal β-glucans

and the galactomannan guar gum carrying a less but still effective structural basis for DC regulatory potential. Of specific notice is the fact that the regulatory pattern found in DC upon interaction with the potent NSP structures concomitant with LPS stimulation, is balanced towards high IL-10 production with very low level IL-12p70 secretion, a DC signature that suggests priming towards a tolerogenic milieu, involving Treg subset generation in the microenvironment surrounding the NSP-triggered DC [188]. Such regulatory profile may be particularly valuable in the highly exposed gut epithelium environment. However, the concomitant induction by some NSP of the pro-inflammatory cytokine TNF-α may disturb polarization into this regulatory Th-cell subset. We did not focus on this issue here, and further studies are therefore needed in order to define the specific functional Th-cell types that are generated by NSP-triggered DC.

Interestingly, a broad range of glycan polymers was observed to hold IL-12p70-inhibitory potential, including so different structural components as cellulose and arabinoxylan. Whether this effect is caused by binding of the rather distinct structures to a single or a number of glucan recognizing receptors on DC, all resulting in IL-12p70 inhibition, cannot be ruled out from the present results. From our data on β-glucans, it was however clear that none of the NSP that inhibited LPS-induced IL-12p70 in DC without simultaneous modulation of other cytokines, stimulated cytokine production in DC by itself, whereas NSP structures that modulated both IL-10 and IL-12 production were all capable to a varying degree of stimulating IL-6 and TNF-α production *per se.* The mechanistic basis for this observation is currently unclear.

Especially the co-stimulatory molecule CD86 on DC was up-regulated by all the β-glucans exhibiting ability to modulate TLR4-induced IL-12p70 and IL-10 production. CD86 engagement with CD28 on T-cells results in T-cell activation provided a simultaneous MHC class II up-regulation takes place [189]. In the presence of LPS, especially lichenan and yeast β-glucan/zymosan exhibited regulatory functions, leading to reduced CD40 and CD80 display along with enhanced CD86 expression on DC. We are currently in the process of evaluating the effect on Th cell polarization of the specific DC phenotype (IL-10(hi), IL-12p70(lo), CD86(hi), CD80(lo), CD40 (interm)) induced by lichenan and yeast β-glucan.

The varying immunomodulatory activity of different β-glucans may relate to the structural basis of the compound. The β-glucans can be divided in groups based on their overall structure: 1) β-glucans containing β-(1,3)-D-glucopyranose units only, 2) β-glucans containing (1,3)- and (1,4)-D-

glucopyranose units, 3) β-glucans containing β-(1,4)-D-glucopyranose units only, and 4) β-glucans with (1,3)- and (1,6)-D-glucopyranose units. Curdlan and paramylon, representative of β-glucans in group 1[183], were observed to possess a medium to strong IL-6 and TNF-α-inducing capacity in DC *per se*, and curdlan additionally induced IL-10 production. This capacity may well be promoted by the LPS present in the curdlan preparation, and the LPS presence here may as well explain the different effects of curdlan and paramylon.

β-glucans from lichenan and cereals both belong to group 2, containing β-(1,3)- and (1,4)-bonds. However, despite their structural similarities, they do not share the same capacity to induce cytokine production or surface marker display in DC. Lichenan exhibited a more potent immunomodulatory capacity than oat- and barley β-glucans. This may be explained by a considerable diversity in the ratio of cellotriose (DP3) to cellotetraose (DP4), the amount of longer cellulose oligomers, and the ratio of (1,4):(1,3) linkages. In general, the ratio of cellotriose- to cellotetraose units for barley β-glucan is 1.8 to 3.5 and for oat β-glucan 1.5 to 2.3, whereas for lichenan β-glucan it is 24.5 [190]. The ratio of the two types of linkages, (1,4) to (1,3), is for both oat and barley between 1.9 to 2.8 [191], whereas lichenan has a ratio of 1.5 to 1.6 [192]. This indicates that β-glucans with a low (1,4):(1,3) ratio and a higher content of cellotriose versus cellotetraose units possess higher immunomodulating capacity, hence, signifying that a greater amount of β-(1,3) glucan structures are central for the immunomodulating potency. This is further supported by the fact that curdlan and partly paramylon, which, together with lichenan, were found to be among the most potent β-glucans tested in the present study, contain β-(1,3) linkages only. In contrast, cellulose that contains β-(1,4) linkages only, did not affect the production of IL-10, TNF-α or IL-6 production, although it did inhibit LPS-induced IL-12p70 production.

The group 4 structures are represented by yeast β-glucan and zymosan, both derived from baker's yeast. Zymosan besides β-glucan also contains mannan [193] that additionally holds immunoregulatory properties [194]. The similar regulatory effects of the galactomannan guar gum and β-glucans on the functional DC cytokine profile indicate that these compounds may interact via the same receptors on DC, or, alternatively, with receptors having identical signaling pathways. Guar gum and β-glucans are recognized to bind to the mannose receptor and also to CR3 (CD11b/CD18) [78] both of which have previously been linked to IL-12 suppression in DC [195].

In this study, we compared a large panel of NSP and consequently we did not focus on identifying the explicit receptor-mediated mechanisms behind the β-glucan and galactomannan effects in DC.

Presently, it is difficult to estimate whether the weaker effect of the cereal-derived β-glucans as in contrast to those of microbial origin is physiologically important, but as the ingestion of plant-derived NSP usually far exceeds that of the β-glucan-containing microorganisms, we cannot exclude that they may play a role in maintaining a tolerogenic milieu in the small intestinal environment. As cereal-derived NSP vary greatly in terms of molecular sizes, we assessed the relevance of the molecular size for immunoregulatory potential by testing β-glucans of varying molecular size generated by enzymic degradation. The cereal β-glucan products differed in cytokine modulating effects and, in general, the products with intermediary molecular weight (25 -215 kDa) displayed activity, while higher and lower molecular weight products did not. The inactivity of the smallest (most degraded) β-glucans may suggest that a certain number of repeats in the pattern of alternating (1,3)- and (1,4)-D-glucopyranose units is required for the β-glucans to modulate the LPS-induced cytokine production. Why the highest molecular weight β-glucan had no effect may be due properties related to solubility or viscosity that may vary with size. In general β-glucans from oat were much poorer immunoregulators than β-glucan from barley. This may be due to variations in the specific structure of β-glucans from these cereals. Other factors relating to specific processes at individual suppliers, such as varieties and growth conditions of cereals used for production, water-holding capacities, and enzymes may also play a role in relation to the immunomodulatory capacity of the products, but our results points towards molecular pattern and size to be major determinants of the immunomodulating activity.

Besides molecular size, one factor that may also influence the immunomodulating potential of NSP is the solubility of the compounds. Some of the tested NSP are insoluble or only slightly soluble under the analytical conditions employed, and there is a great variability within the different preparations. In order to take into account both soluble and insoluble parts, we here suspended all products in growth medium, and soaked the preparations for 1 hour before adding a representative suspension of the sample to cells. Based on this approach, we cannot decipher whether the solubility of a given product is of essential importance for the bioactivity. However, in a pre-study where DC was treated with the soluble fraction only, we found a reduced or no modulatory effect, suggesting that solubility is not a prerequisite for the bioactivity. We cannot, however, from the

present study conclude whether solubility is a critical parameter for immunomodulationg of NSP *in vivo.*

Conclusively, we here demonstrated that, amongst the various NSP products tested, superior DC immunomodulatory activities are found within the group of β-glucans, and in the galactomannan guar gum. Comparisons between different cereal-based β-glucans made it clear that these products vary greatly in terms of bioactivity, and that size may be of specific importance in relation to the immunoregulatory properties of the product. Enhanced insight into the structural requirements for NSP efficacy, such as set forth in this larger comparative study, may facilitate development of more effective NSP-based products and promote their use as dietary supplements.

CHAPTER 4

β-GLUCAN-INDUCED PHENOTYPIC PROFILE OF TOLL-LIKE RECEPTOR-TRIGGERED DENDRITIC CELLS

In collaboration with Susanne Brix

The phenotype of dendritic cells (DCs) determines the outcome of adaptive T cell responses and therefore the pathogen-specific phenotypic signature in DCs plays a significant role in regard to efficient clearance of intruding pathogens. β-glucans are structural cell wall components in fungi and a few plants, and are recognized by specific C-type lectin receptors (CLRs). We show here that β-glucans of diverse origins modify toll-like receptor (TLR)-induced response patterns in human monocyte-derived DCs, and imprint an elementary change in the functional phenotype. The β-glucan-modified DCs were dispersed into two subsets, one with low level CD40 and CD86, and the other with high level CD86 and intermediate CD40 display. On a general level, the TLR-triggered DCs enhanced the production of CCL4, IL-10, IL-2, IL-1β, CXCL8 and TNF-α, while the level of CXCL10 was reduced by all β-glucans. Modification of TLR-triggered IL-6, IL-23, IL-12p70 and CCL17 levels varied dependent on the β-glucan origin. Expression levels of the CLRs Dectin-1, DC-SIGN and the mannose receptor were down-regulated differentially according to the β-glucan origin. β-glucan mediated modification of TLR-triggered DCs significantly affects the functional phenotype of DC, and therefore β-glucans may potentially twist polarization of DC-induced $CD4^+$ T cell responses in an inflammatory setting.

INTRODUCTION

The initial recognition and uptake of antigen by antigen presenting cells (APC) such as dendritic cells (DCs) is essential for immunological elimination of pathogens [196]. Immature DCs senses its surroundings and captures antigens by several mechanisms, and upon capture of pathogens, DCs undergoes phenotypic changes, which allow them to migrate to the lymph nodes. Here they prime T cells to an appropriate response, depending on their phenotype [197].

The polarization of a T cell during activation by DC is dependent on the type of molecules engaged by the DCs. In order to distinguish and react differently to a large array of foreign compounds, DC expresses several classes of pattern recognition receptors (PRR). PRR recognizes highly conserved molecular patterns presents in microorganisms known as pathogen-associated molecular patterns (PAMP). PRRs important for DC recognition of foreign molecules include e.g. the Toll-like receptors (TLR) and C-type lectin receptors (CLR) [198].

The hallmark of CLRs is recognition of specific carbohydrate structures [22]. For instance, Dectin-1 recognizes β-glucan exposed on fungi and yeast, such as *Candida albicans* and *Saccharomyces cerevisiae* [199, 200], whereas DC-SIGN and the mannose receptor (MR) are shown to interact with mannose-containing carbohydrates present on e.g. *C. albicans* [201], HIV-1 [202], and *Mycobacterium tuberculosis* [203], and also has affinity for fucose-containing glycans [24, 25].

CLRs on immature DCs play a role in its antigen capture function. Upon carbohydrate-mediated binding, CLRs are involved in the internalization of pathogens and/or molecules thereof leading to processing and presentation of antigenic peptide subunits onto MHC class I or II molecules [204]. The role of CLRs in immune modulation is yet largely uncovered. It has previously been described that activation of CLRs leads to initiation of signaling cascades that involves activation of the transcription factor κB (NF-κB), which leads to expression of genes involved in maturation of DCs and the ability to prime and skew T cell responses [205]. However, it is still elusive whether or which CLRs that hold capacity to act directly and enhance expression of certain gene products, or whether they affect DC expression profiles via modification of TLR-induced pathways. The presence of TLR-ligands in several well-studied products of, for instance, β-glucans, such as zymosan (lipoproteins [206]) and curdlan (LPS [207]), points to a possible crosstalk between TLR-

CLRs in mediating the signal modification in DCs. Several data by other groups supports this notion, including Geijtenbeek and colleagues showing that the interaction of mannose-containing compounds with DC-SIGN affects TLR4-mediated immune responses by DC [84, 208]. The crosstalk was found to integrate at the level of TLR-dependent NF-κB, and NF-κB activation induced by other PRRs, like TLR3 and 5, are likewise affected [208]. Similarly to DC-SIGN, the β-glucan receptor Dectin-1 [204], and MR [173] was also shown to modulate TLR-induced NF-κB. Currently, we lack insight into how the phenotype of DCs is modulated by CLR-triggering factors when present in combination with TLR-triggering ligands. To increase our insight into glucan-induced modulation of the DC phenotype, we here studied one glucan family, the β-glucans that have attained great focus due to their specific recognition by phagocytes, such as DCs, and their presence in pathogenic and non-pathogenic fungi, and in diverse edible and non-edible plants.

In order to describe the distinct differences in-between β-glucans of diverse origins on modification of a TLR-triggered DC phenotype, we here examined and compared the response pattern from DCs upon simultaneous triggering with the TLR4-ligand lipopolysaccharide (LPS). We here examined the activation and involvement of the CLRs; Dectin-1, DC-SIGN and MR in mediating β-glucan effects. Moreover, we measured an array of cytokines, chemokines and surface markers on DCs that enabled us to designate a general β-glucan-induced DC phenotype.

MATERIALS AND METHODS

Cell isolation and culture

DCs were generated from PBMC as described by Zhou and Tedder with minor modification [209]. Briefly, PBMC were isolated from buffy coats (Copenhagen University Hospital, Denmark) by density gradient centrifugation in Ficoll-Paque (GE Healthcare Biosciences, Uppsala Sweden). The PBMC were washed twice with RPMI 1640 (Lonza, Verviers, Belgium) added 100 U/mL penicillin 100 μg/mL streptomycin (Lonza), and monocytes were isolated by magnetic activated cell sorting (MACS) with $hCD14^+$ microbeads (Miltenyi Biotech, Bergish Gladbach, Germany). The monocytes were cultured at a density of $6 \cdot 10^6$ cells/3 ml/well in six-well tissue culture plates for 6 days in culture medium (RPMI 1640 supplemented with 2 mM L-glutamine (Lonza), 100 U/mL penicillin 100 μg/mL streptomycin (Lonza), 50 μM 2-mercaptoethanol (Invitrogen, Carlsbad, CA), 10 % heat inactivated fetal bovine serum (FBS)(Lonza)) containing 30 ng/ml recombinant human

IL-4 (Invitrogen) and 20 ng/ml recombinant GM-CSF (Invitrogen). After 3 days incubation, 1 ml was removed and replaced with 1.5 ml fresh culture medium added IL-4 and GM-CSF. After 6 days, the $CD14^+$ cells were differentiated into non-adherent immature DC (iDC).

Stimulation of DC

On day 6, the iDCs were pooled, harvested (by centrifugation at 280x g for 10 min) and reseeded in 48-well tissue culture plates (Nunc) at $6 \cdot 10^5$ cells/600 μl/well. DCs were cultured with β-glucans at a final conc. of 100 μg/mL. LPS from *Escherichia coli O26:B6* (Sigma-Aldrich Inc. St. Louis, MO) was added to a final conc. of 1 μg/mL. Cells added medium alone were used as negative control. The cells were incubated for 18 h at 37°C in 5 % CO_2. The cultures supernatants were collected and stored at -20 °C until cytokine analysis, and cells were harvested for flow cytometric analysis.

Cytokine quantification in culture supernatants

The production of IL-10, IL-12p70, IL-12p40, and TNF-α were analyzed using commercially available ELISA kits (R&D systems, Minneapolis, MN) according to the manufacturer's instruction. IL-23p19/p40 was analyzed using a commercially available ELISA kits (eBioscience, San Diego, CA). For multiplex analysis, the levels of IL-1β, IL-2, IL-10, IL-12p70, TNF-α, CXCL8(IL-8), CCL11(eotaxin-1), CXCL10(IP-10), CCL2(MCP-1), CCL13(MCP-4), CCL22 (MDC), CCL4(MIP-1β) and CCL17(TARC) were analyzed in the supernatant using the Meso Scale Discover multiplexed array system (ultrasensitive Human 10-plex Th1/Th2 cytokine assay and 9-plex chemokine assay). Assays were conducted according to standard manufacturer's protocols and samples were read using the Sector Imager 6000 (Meso Scale Discovery, Gaithersburg, MD, USA). The sensitivities for all cytokines were typically below 3 pg/ml and chemokines ranged between 1-230 pg/ml. The lower level of detection: IL-1β = 0.4 pg/ml, IL-2 = 1.3 pg/ml, IL-10 = 0.86 pg/ml, IL-12p70 = 2.21 pg/ml, TNF-α = 0.54 pg/ml, CXCL8 = 0.69 pg/ml, Eotaxin-1 = 11.5 pg/ml, CXCL10 = 46.7 pg/ml, CCL2 = 2.67 pg/ml, CCL13 = 11.8 pg/ml, CCL22 = 228 pg/ml, CCL4 = 3.82 pg/ml and CCL17 = 228 pg/ml.

Immunostaining and flow cytometry

After collection of supernatant, ice cold PBS-az (PBS containing 1% (v/v) heat-inactivated FBS and 1.5% (w/v) sodium azide (Sigma-Aldrich Inc.)) was added to prevent internalization of surface markers during subsequent handling of the cells, and the cells were kept at 4 °C or below and under

low level of light. Non-adherent cells were collected by gentle pipetting and transferred to 96-well plates. To block non-specific binding of Abs, the cells were incubated for 5 min with 25 μL/well PBS-az containing 10% human AB serum (Copenhagen University hospital, Denmark).

After incubation, additional 25 μL/well PBS-az containing fluorochromes-conjugated antibodies was added and incubated for 45 min. Subsequently, the cells were washed twice in 150 μL/well PBS-az and resuspended in PBS-az for flow cytometry analysis in a FACScanto II bioanalyser (BD Bioscience). The analysis was based on 30,000 cells. The following antibodies were used: FITC conjugated anti-human DC-SIGN, clone DCN46, mouse IgG2b (BD Bioscience); PE conjugated anti-human Dectin-1, Clone 259931, mouse IgG2b (R&D systems); PerCP-Cy5.5 conjugated anti-human CD40, clone 5C3, mouse IgG1 (Biolegend, San Diego, CA); V450 conjugated anti-human CD86, clone 2331 (FUN-1), mouse IgG1 (BD Bioscience); APC conjugated anti-human CD206, clone 19.2, mouse IgG1 (BD Bioscience). Isotype controls for: APC conjugated mouse IgG1, FITC conjugated mouse IgG2b, V450 conjugated mouse IgG1 (all BD Bioscience), PE conjugated mouse IgG2b (R&D systems), PerCP-Cy5.5 conjugated Rat IgG2a (Biolegend).

Data were analyzed using FCS Express (version 3.0, De Novo Software, Los Angeles, CA).

Statistical analysis

The data were analyzed for statistical significance (GraphPad Prism, version 5.01, GraphPad Software, San Diego, CA) using one-way ANOVA and the Tukey post test. A P-value < 0.05 was considered significant (*), $P < 0.01$ = ** and $P < 0.001$ = ***.

RESULTS

In this study, we examined the dynamic regulation of a TLR-triggered phenotype in human monocyte-derived DC by distinct β-glucans. The β-glucans employed in this study was of microbial and plant origins and differed in structures, purity and contamination with TLR- and CLR-ligands as detailed in Table 1. β-glucan preparations contain varying amounts of TLR-triggering ligands, dependent on their origins, and are generally difficult to purify for TLR-ligand contaminants prior to cellular experiments due to their insoluble nature in aqueous solutions. These properties make it difficult to perform comparative analysis of the potency of different β-glucan structures, and to interpret specific mechanisms of action of β-glucans *per se*. Here we opted to study the TLR modifying properties of structurally diverse β-glucans, and therefore we added the TLR-4 ligand

LPS simultaneously with all β-glucan preparations, hence applying at least one TLR ligand to every treatment.

Table 2: β-glucan structures, origin and known content of TLR- and CLR-ligands.

	Chemical Structure	Origin	Purity (%)	Known TLR-contaminants	Detected CLR-ligands[1]
Zymosan	1,3-1,6-β-D-glucan	*Saccharomyces Cerevisiae*	n.a.	Lipoproteins [206]	9.9 % Mannan
Yeast β-glucan	1,3-1,6-β-D-glucan	*Saccharomyces Cerevisiae*	> 90	n.a.	2.0 % Mannan
Lichenan	1,3-1,4-β-D-glucan	*Cetraria islandica*	> 85	n.a.	1.6 % Mannan and 1.4 % Galactose
Curdlan	1,3-β-D-glucan	*Alcaligenes faecalis*	> 99	LPS [207]	n.a.
Barley β-glucan	1,3-1,4-β-D-glucan	Barley	n.a.	n.a.	n.a.

1: Own data, given as % of dry-matter
n.a.: not available

Modulation of Dectin-1, DC-SIGN and Mannose receptor expression by diverse β-glucans

Initially, the effect of β-glucans on the expression levels of the three CLRs; Dectin-1, DC-SIGN and the mannose receptor (MR) was evaluated. It is generally acknowledged that iDCs expresses the highest levels of CLRs, and that activated, mature DC internalizes the CLR upon interaction with pathogens or pathogenic ligands. By comparing the response pattern to an array of β-glucan molecules, we here observed that reduction in display of the individual CLRs differs in-between the three receptors, and depends on the origin of the ligand (Fig. 1). A large proportion (87-89%) of viable immature DC were found to express the β-glucan receptor Dectin-1 as well as the mannose- and fucose-recognizing receptors, MR and DC-SIGN (Fig. 1); and with all positive iDCs expressing all three CLRs simultaneously (Fig. 1a-c). Upon LPS addition, the same expression levels were observed for DC-SIGN and MR, while a 30% reduction in the Dectin-1 expression was observed as compared to iDC expression levels (Fig. 1a, d). All β-glucan-containing products were found to down-regulate the surface display of Dectin-1 by more than 50% in TLR4-triggered DCs as in contrast to DCs cultured with LPS alone (Fig. 1d), indicating that Dectin-1 is being cross-linked by the structurally diverse β-glucans. We found that overall MR display was down-regulated by lichenan, zymosan and yeast β-glucan (Fig. 1d), which is most probably due to presence of alpha-

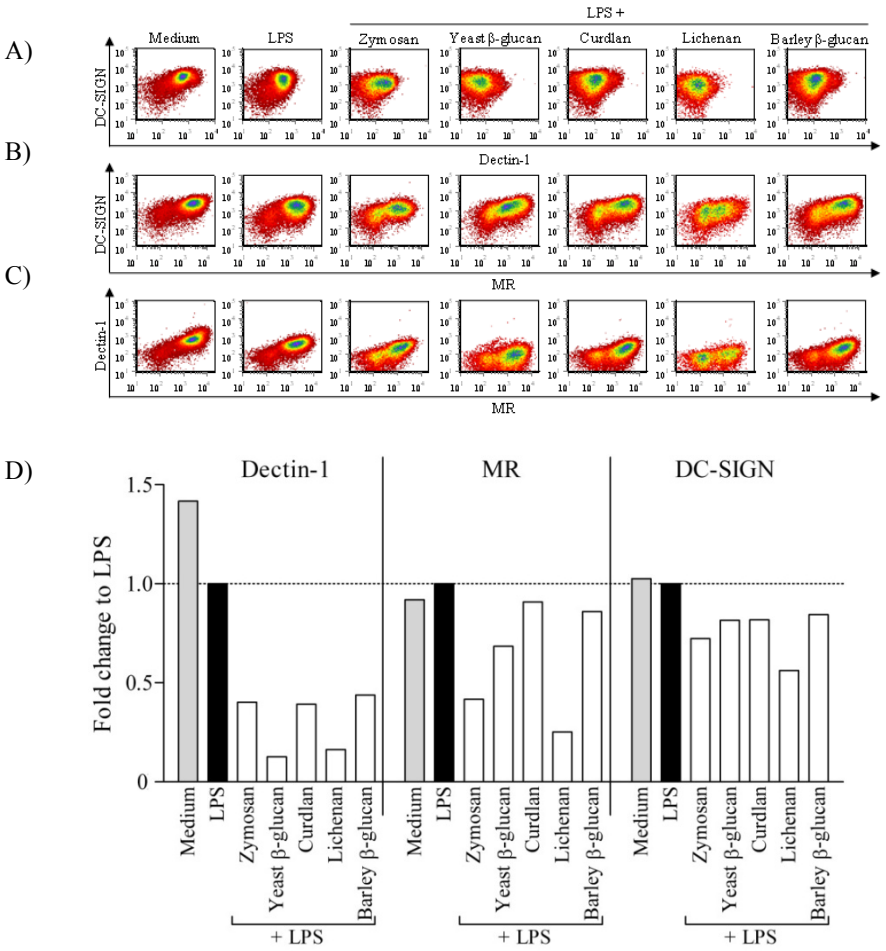

Figure 1. **β-glucan-mediated modulation of Dectin-1, mannose receptor and DC-SIGN display in TLR-triggered DC.**
Human monocyte-derived DC were cultured for 18 h with β-glucan containing compounds (200 μg/ml) in the presence of LPS (1 μg/ml) or with medium alone. The expression levels of the CLRs Dectin-1, the mannose receptor (MR) and DC-SIGN were determined by flow cytometry after staining with fluorescence-conjugated antibodies. **A-C**: Density plots displaying the β-glucan mediated regulation of the three CLRs in the presence of LPS. Cells were gated on viable CD11c positive cells, and 30.000 cells are displayed. **D**: Comparison of the β-glucan mediated regulation of the cellular expression of the three CLRs given as a ratio to LPS-induced expression levels. The data represents results from one donor, and is comparable to the data from three other donors.

1,2-mannan in the lichenan and zymosan products, and also in yeast β-glucan, although to a minor extent (Table 1). It is well known that zymosan is containing mannan residues due to the presence of mannan in the yeast membrane, but it is not well documented that lichenan- and yeast β-glucan products are able to co-activate MR and Dectin-1. Curdlan and barley β-glucan were not found to activate DC through the MR (Fig. 1d). DC-SIGN was regulated by all β-glucans with surface display being reduced with 15-45%, and with the largest effect observed for lichenan. The regulatory pattern of DC-SIGN was dissimilar to that of Dectin-1, and more alike that of the MR, implying that diverse structures regulate the three CLRs, but with more structural similarity

between the ligands for MR and DC-SIGN than for Dectin-1. The present data on CLR regulation by diverse β-glucan-containing compounds demonstrates that Dectin-1, MR and DC-SIGN are regulated in a ligand-receptor specific manner, also upon TLR-induced activation, and that only Dectin-1 is subject to change in surface expression levels upon TLR4-triggering of DCs.

Distinct patterns of cytokine and chemokine modulation in TLR-triggered DC by β-glucans

Based on the diversity of CLR regulation by the diverse β-glucan structures, including the microbial-derived β-glucan curdlan, earlier reported to induce Th17 cells [210], we compared the β-glucan-induced pattern of DC-derived cytokines of importance for differentiation and expansion of naïve Th into the specific functional subsets; Th1, Th17 and Treg. These studies were performed in order to increase our insight into the diversity in-between β-glucans in terms of their modulation of the Th cell polarizing properties of DCs. The DCs were activated with LPS, and the ability of the five different β-glucans to modulate the production of IL-12p70, IL-23, IL-12p40 and IL-10 was measured upon 18h of incubation. IL-10 has the potential to suppress an immune response, especially T cell responses, by down-regulating pro-inflammatory immune reactions, and enhancing Treg development [211, 212], whereas IL-12p70 and IL-23 are associated with Th1 and Th17 differentiation and expansion, respectively [213, 214]. IL-12p40 is a prerequisite for IL-12p70 and IL-23 production, as IL-12p40 dimerizes with IL-12p35 to form bioactive IL-12 (IL-12p70), or with IL-23p19 to form IL-23.

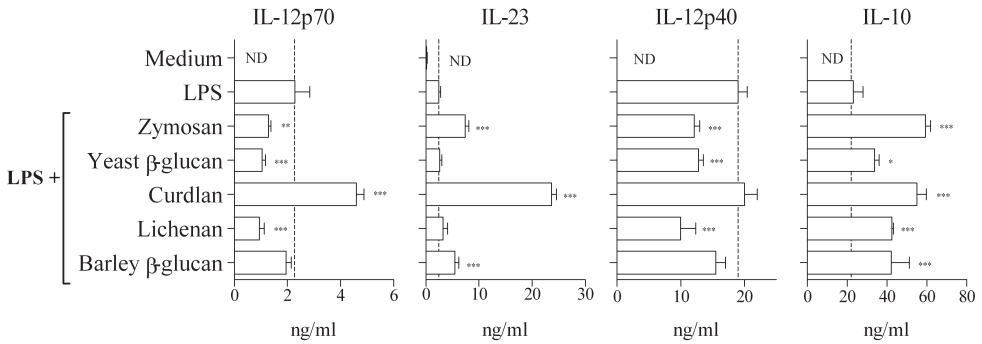

Figure 2. Distinct modulation of IL-12p70, IL-23, IL-12p40 and IL-10 in TLR-triggered DC by β-glucans of diverse origins.
Cytokine levels as measured in culture supernatant from human monocyte-derived DC upon culturing for 18h with indicated β-glucan-containing compounds (200 μg/ml) in the simultaneous presence of LPS (1 μg/ml), or with LPS or medium alone. The dashed line represents cytokine production from LPS-treated DC. The levels of cytokines were determined by ELISA. Data are mean + SD, and represents data from 3 individual experiments. Differences between dual-treated DC as compared to LPS-treated DC were tested by one-way ANOVA and the Tukey post-test. $P < 0.05$, *, $P < 0.01$, **, $P < 0.001$. ***.

Several of the β-glucans including lichenan, zymosan and yeast β-glucan were capable of suppressing TLR-induced IL-12p70 production, whereas curdlan showed IL-12 p70-inducing capacities (Fig. 2). Interestingly, the IL-23 secretion pattern was opposing to that of IL-12p70 for most β-glucan-containing products, as except for curdlan that induced expression of both cytokines (Fig. 2). The levels of IL-12p40 were regulated like IL-12p70 by the different β-glucans, besides for curdlan where IL-12p40 levels were similar to that of LPS. Thus, the β-glucans varied significantly in their IL-12p70/IL-23-modifying competences, with curdlan being able to induce both IL-12p70 and IL-23 simultaneously, while yeast β-glucan and lichenan reduced IL-12p70 without enhancing IL-23, and zymosan reduced IL-12p70 and augmented IL-23. Barley β-glucan increased IL-23 secretion only. In contrast to the variability in IL-12p70/IL-23 secretion patterns between the β-glucans, the IL-10 production was found to be increased by all β-glucans.

To increase our general knowledge on the 'secretory fingerprint' from β-glucan modified TLR-triggered DC that play a role for Th cell polarization and recruitment, we measured eight different chemokines (CXCL10, CCL22, CCL17, Eotaxin, CCL4, CCL2, CCL13 and CXCL8) and four other cytokines (IL-6, IL-1β, IL-2 and TNF-α), besides IL-12p70, IL-12p40, IL-23 and IL-10. The DC produced chemokines attracts other cells of the immune system. CCL2 and -4 are known to recruit monocytes [215], and CXCL8 attracts neutrophiles [216], while the other measured chemokines recruit certain Th effector cells: CXCL10 (Th1), CCL22 (Th2), CCL17 (Th2), Eotaxin (Th2), and CCL4 (Treg) [217-219]. IL-2 is necessary for proliferation of T cells, and plays a specific role for the proliferation of Tregs, due to their high expression of CD25 [44]. TNF-α, IL-6 and IL-1β are pro-inflammatory factors with the latter being produced upon activation of the inflammasome [220], and with IL-6 and IL-1β being necessary for differentiation of naïve human T cells to Th17 [221].

Generally, the β-glucans showed a similar capacity to regulate chemokine production in TLR-triggered DC with suppression of CXCL10, CCL22, CCL17 and CCL13, and enhancement of CCL4 and CXCL8 (Fig. 3a), but curdlan tended to diverge from the common suppressive pattern and induced a more LPS-alike CCL22, CCL17 and CCL13 response. For Eotaxin and CCL2 only minor effects were observed for lichenan and zymosan (Fig. 3a). A general β-glucan-induced chemokine profile in TLR-induced DC is characterized with CXCL8(hi), CCL4(hi), CCL13(lo), CXCL10(lo) and CCL17(lo).

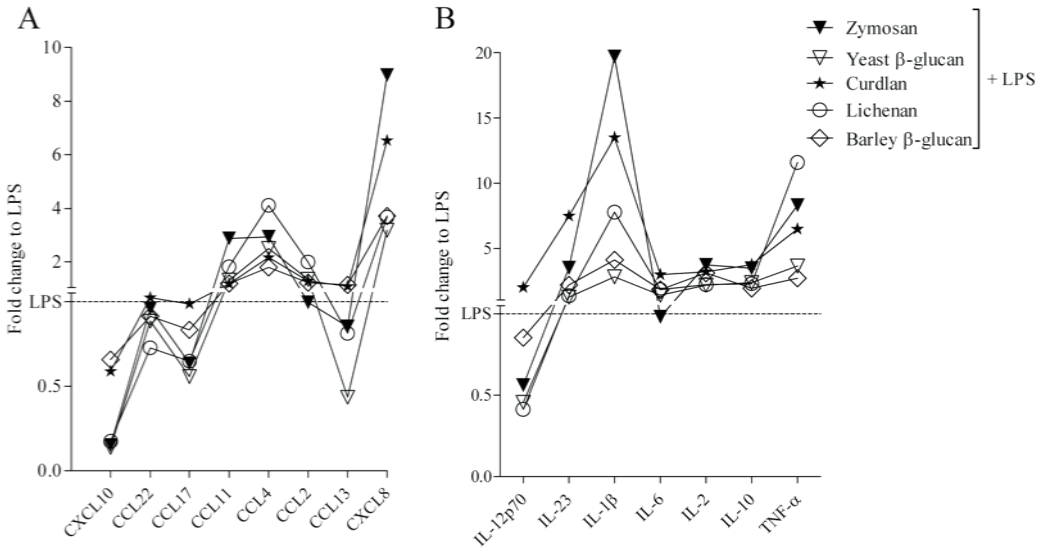

Figure 3. β-glucan-induced modification of the secretory profile in TLR-triggered DC.
Selected regulatory chemokines (**A**) and cytokines (**B**) were quantified by ELISA in culture supernatants from human monocyte derived DC upon culturing for 18 h with the different β-glucan compounds (200 μg/ml) in the presence of LPS (1 μg/ml), or with LPS alone. The dashed line represents cytokine production from LPS-treated DC. Data are presented as the fold change to LPS stimulation alone, and represent results from one donor out of four. For simplicity the SD is not included in the charts, but they equal those presented in fig. 2 for four of the cytokines (IL-12p70, IL-23, IL-12p40 and IL-10). The values for LPS-treated DC were: IL-2: 690 pg/ml, IL-6: 25.1 ng/ml, IL-10: 25.5 ng/ml, IL-12p70: 2.50 ng/ml, TNF-α: 41.3 ng/ml, IL-23: 4.31 ng/ml, IL-1β: 161 ng/ml, CXCL10: 52.4 ng/ml, CCL22: 145 ng/ml, CCL17: 45.6 ng/ml, CCL11: 2.85 ng/ml, CCL4: 81.7 ng/ml, CCL2: 1.03 ng/ml, CCL13: 2.96 ng/ml and CXCL8: 106 ng/ml.

Contrarily to regulation of the chemokines, most cytokines were observed to be enhanced by the β-glucans in TLR-triggered DC, besides for IL-12p70 that was reduced upon zymosan, yeast β-glucan, lichanen and barley β-glucan treatment (Fig. 3b). The production of IL-2 was similar to that of β-glucan-induced IL-10 and IL-23 levels (Fig. 3b), whereas TLR-triggered TNF-α and IL-1β were induced to higher levels than IL-10, IL-2 and IL-23 upon treatment with lichenan, zymosan or curdlan. IL-6 secretion levels were not induced significantly by the β-glucans, except for curdlan that enhanced levels three-fold (Fig. 3b).

Collectively, the enhancement of TLR-triggered IL-1β, IL-23, IL-10, IL-2, CCL4, CXCL8 and inhibition of CXCL10, CCL17 and partly IL-12p70 secretion patterns point toward a β-glucan-induced DC profile that inhibits Th1, and perhaps Th2 development (CCL17 suppression), while promoting Th17 and/or Treg development.

Diverse regulation of CD86 and CD40 expression by β-glucans in TLR-triggered DC

To further describe the regulatory profile of DC upon β-glucan engagement, the levels of changes in expression of the surface molecules CD40 and CD86 were measured. CD40 is important for the DC-T cell crosstalk and activation of Th1 type responses [222], whereas CD86 play an outmost role in activation of T cells via interaction with CD28, however, its specific role in T cell differentiation is currently not fully disclosed [223].

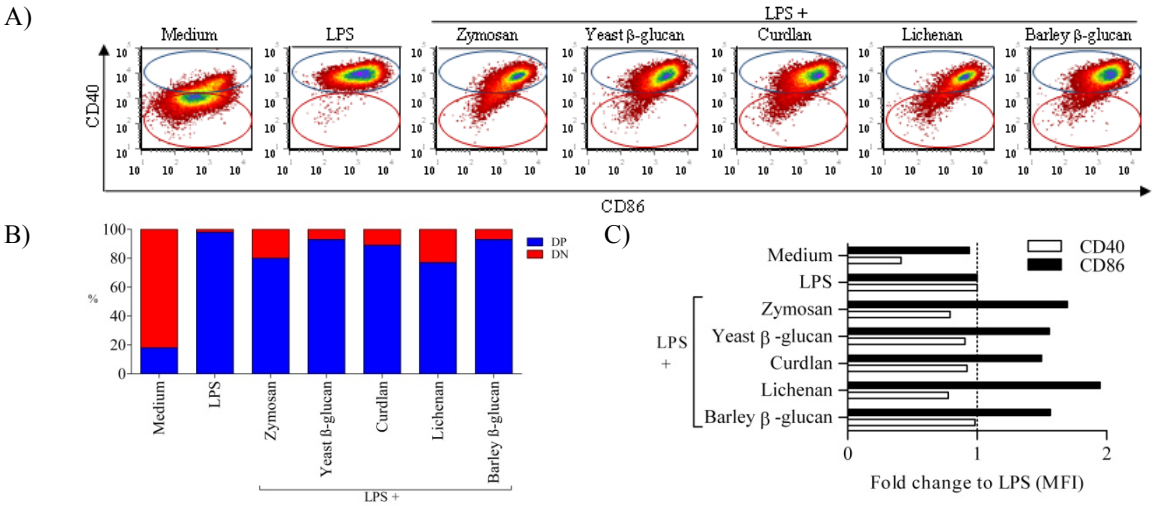

Figure 4. β-glucan-mediated modulation of CD86 and CD40 display on TLR-triggered DC.
Human monocyte-derived DC were cultured for 18 h with different β-glucans (200 μg/ml) in the presence of LPS (1 μg/ml), or with LPS or medium alone. The expression levels of CD40 and CD86 were determined by flow cytometry after staining with fluorescence-conjugated antibodies. Cells were gated on the viable CD11c population, and a total of 30.000 cells within this gate were recorded. **A**: Density dot plots. The cells were divided into two subpopulations consisting of $CD86^{+}CD40^{+}$ (double positive, DP) and $CD86^{-}CD40^{-}$ (double negative, DN). **B**: Percentage of cells in DP versus DN from A) for each DC treatment. **C**: Comparison of the cellular expression level in the DP cell population (given as mean fluorescence intensity (MFI)) of the CD40 and CD86 expression levels for each b-glucan treatment as a fold change to that of LPS-treated DC. Data represents one out of four experiments with cells from different blood donors.

TLR4-mediated activation of DC resulted in up-regulation of CD40 and CD86 with 99% of viable DC displaying both surface markers (double positive, DP, Fig. 4a,b) as compared to untreated, immature DC where 80% of all cells are double negative (DN, Fig. 4a,b). All β-glucans were able to modulate the expression of the TLR-induced display of CD40 and CD86 with two subpopulations of DC being generated upon interaction with β-glucans. A DN subset representing 10-23% of the DC population that inhibits LPS-induced CD40 and CD86 display, hence representing a DC subpopulation with immature-like properties, and a diversely regulated subset, representing 80-90% of the DCs, with enhanced CD86 expression, and a slightly reduced CD40

display as compared to TLR4-treated cells (Fig. 4c) that may correspond to a subtype of DC that induces Th17 differentiation.

β-glucan-induced phenotypic profile in TLR-triggered DC

To compare the overall phenotypic profiles induced by the different β-glucan products in TLR-triggered DC, a heatmap was generated displaying the individual protein levels relative to LPS-treated DCs within each molecular subgrouping (Fig. 5). A common phenotypic profile of β-glucan-modified DC is clearly emerging from this comparison, revealing that the molecular signature that best describes a general β−glucan-induced profile is enhancement of TLR4-induced CCL4, CXCL8, IL-1β, IL-2, IL-10, TNF-α and CD86, and suppression of CXCL10 and CD40 (Fig. 5). The suppression of the TLR-triggered levels of CCL17, CCL22, CCL13 and IL-12p70, and the enhanced levels of IL-23 and IL-6 seems to be dependent on the β-glucan origin; i.e. the glycan structure or the endogenous TLR- or other CLR-ligands, besides the β-glucan that are present in all products.

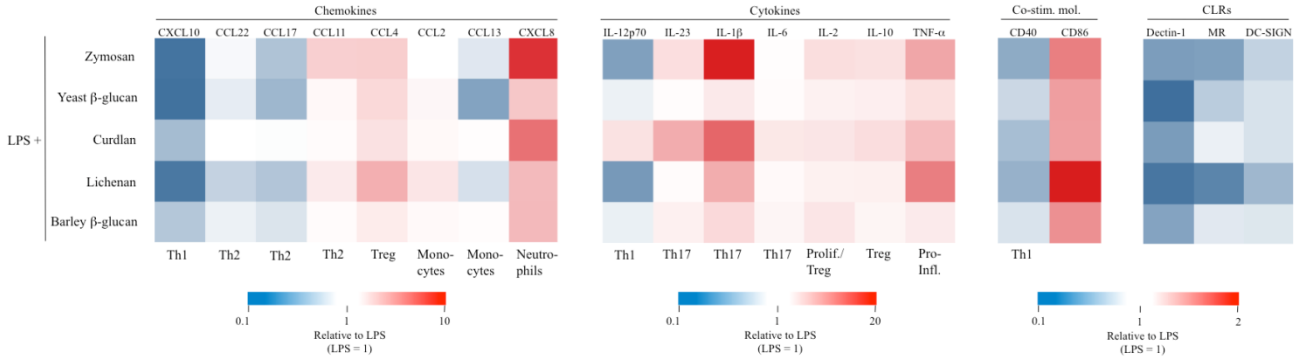

Figure 5. Comparison of the phenotypic profile induced by different β-glucans in TLR-triggered DC.
The heatmap is displaying the levels of DC expressed cytokines, chemokines, co-stimulatory molecules and the CLRs as compared to LPS (representing a value of 1). Blue and red colors represent expression levels lower and higher, respectively to LPS-pulsed DC. Color intensity is scaled within each subgrouping so that the highest expression value corresponds to bright red and the lowest to bright blue. The heatmap displays data from one donor, and is representative for the expression pattern in four donors. (Prolif. = Proliferation, Pro-infl. = Pro-inflammatory)

Moreover, it also appears from this comparison that barley β-glucan is less capable of modifying the TLR-triggered response pattern as compared to the microbial-derived β-glucans from zymosan and yeast β-glucan, as well as the plant-derived β-glucan lichenan. Noticeably, curdlan is found to deviate from the other β-glucans in its IL-23, IL-6 and IL-12p70-inducing properties, and absence of CCL17, CCL22 and CCL13-suppressive capacity, hence generating another phenotypic profile in

TLR-triggered DC than observed for zymosan, yeast β-glucan and lichenan. The expression levels of the three CLRs, Dectin-1, MR and DC-SIGN, were all down-regulated upon glycan interactions.

DISCUSSION

The phenotype of DCs determines the outcome of adaptive T cell responses and due to this feature the pathogen-specific phenotypic signature in DCs plays a significant role in regard to appropriate instruction of T cell responses as to efficiently clear off intruding pathogens. Phenotypic regulation of DC is exerted through expression of a range of different PRRs that mediate structure-dependent interactions with surrounding compounds. Pathogens harbor molecules that interact with several known PRRs including TLRs and CLRs, but yet we lack insight into how simultaneous cross-linking of TLRs and CLRs by concurrent presence of various pathogenic molecules directs the DC phenotype. In this study, we used a simple model system to deduce the molecular phenotype of DC upon interaction with the fungal and plant-related CLR-ligand, β-glucan, when present in conjunction with at least one TLR stimulus, LPS from *E. coli*, added exogenously to all treatments.

Our present work demonstrates that β-glucans indeed modify a TLR4-induced activation profile in human monocyte-derived DC. The general DC phenotype that arises is principally based on high level expression of CCL4, IL-10, IL-2, IL-1β, CXCL8, TNF-α, and low level CXCL10, with varying levels of IL-6, IL-23, IL-12p70 and CCL17 dependent on the origin of the β-glucans. An evaluation of the CD40 and CD86 expression levels showed that the β-glucan-modulated DCs was distributed into two subsets, one with low level CD40 and CD86, suggestive of a regulatory-like subset, and one with high level CD86 and intermediate CD40 display. The CD40 (lo), CD86 (lo) subset may represent a tolerogenic DC subset that mediate priming towards Treg generation [188] (based on production of CCL4, IL-10, IL-2, in combination with low levels of CD40, CD86 and CXCL10, and potentially also of IL-12p70, CCL22 and CCL17), whereas the CD86 (hi), CD40 (int) may prime and propagate Th17 cells (IL-6, IL-1β, IL-23, CD86 and CD40). However, more studies are needed in order to verify the specific outcome of the β-glucan-mediated DC phenotype on polarization and activation of naïve Th cells. Noticeably, however, the effect of curdlan on the DC regulatory pattern was shown to be different than that of most other β-glucans emphasizing that great care should be taken when extrapolating data on specific β-glucan preparation properties to represent that of β-glucans, and also fungi, in general. It is in general difficult to address the endogenous immunoregulatory effect of β-glucans, due to the presence of various CLR-ligands and

also TLR-ligands in the commercial preparations that are commercially available. Curdlan excels from the other β-glucans in its ability to induce concomitantly high levels of IL-23 and IL-12p70, and yet showing a poor ability to induce CXCL10 and to increase the TLR-induced CD40 surface display of importance for Th1 and Th17 generation [222, 224, 225]. Curdlan has previously been associated with a Th17-inducing property in well-documented *in vitro* and *in vivo* studies by acting via a Dectin-1-dependent mechanism [226, 227]. This property is well supported by our current data showing a phenotypic cytokine and chemokine profile in DCs with high level IL-1β, IL-6 and IL-23, and low level CXCL10 [228, 229], along with display of the co-stimulatory molecules CD86 and CD40. Despite that curdlan is mediating a slightly different phenotypic profile in DC than observed for the other β-glucans, it is not evident from a comparison of their fingerprints that the resulting Th polarization properties would be notably different, as a suppression of IL-12p70 together with high levels of IL-1β by the other β-glucans may end up having the same effects. We have unpublished data from mice DC showing that all β-glucans hold the Th17-polarizing property although curdlan is the most potent one, and are currently in progress with studies to confirm these data in human DC. The curdlan was, contrarily to the other β-glucans, found to contain detectable levels of endotoxin, i.e. LPS, with 100 endotoxin units pr mg corresponding to roughly 20 ng LPS/mg [207]. This level of LPS in curdlan may contribute to enhance the IL-12p70 production in DC, but as other potent β-glucans, such as zymosan, reduced LPS-triggered IL-12p70 production, the LPS, other unknown contaminants, or the lack of e.g. mannan in the curdlan preparation seems to affect the LPS-triggered response pattern in a diverse manner than seen for the other β-glucans. Curdlan is derived from the fecal Gram-negative bacterium, *Alcaligenes faecali*, and the differences in properties of curdlan as opposed to the other β-glucans may also be based on its molecular structure (1,3-β-glucan).

Our data on CD40 and CD86 expression levels points to the presence of two β-glucan-induced DC subsets with different phenotypes; a minor population of representing a ‘regulatory’-alike phenotype with low level expression of CD40 and CD86, and a larger fraction of ‘inflammatory’-alike DCs with enhanced CD86 expression levels and intermediate levels of CD40 as compared to LPS-triggered DC. Down-regulation of CD40 is associated with a regulatory DC phenotype [230], whereas a reduced level of CD86 display will prevent efficient co-stimulation of T-cells. The lack of production of chemokines associated with recruitment of Th1/Th2 cells (CXCL10, CCL17, CCL22), and enhanced secretion of Treg attractant chemokines (CCL4), as well as IL-10 and IL-2

further supported that a subset of tolerogenic DCs may be induced by β-glucans. That the dominant subpopulation (CD86hi, CD40int) may represent a Th17-polarizing DC subset is speculative; since we are still short of data that describes the phenotype of Th17-polarizing DCs. Th17 cells are reported to express a range of chemokine receptors (CCR2, CCR4, CCR5, CCR7, CXCR3 and CXCR6) [231], and therefore the chemokine profile that promotes their migration is yet elusive. However, both CD86 and CD40 have been described to promote Th17-differentiation [232], and moreover, the cytokine pattern from the β-glucan-modified TLR-triggered DC showed induction of IL-1β, IL-23, concomitant with high IL-6 levels, suppression of CXCL10 (and IL-12p70), and an increase in IL-10 that may limit the Th1 skewing potency, implying a Th17-promoting DC phenotype. Curdlan and *C. albicans* have previously been reported to promote Th17-responses in mouse DC and upon *in vivo* injection, respectively [226], and based on these studies and our current data it is likely that β-glucans are involved in promoting Th17-differentiation by modifying the TLR-triggered DC phenotype. In relation to the tolerogenic DC phenotype, it was previously shown by Dillon *et al.* [128] that zymosan can promote immunological tolerance in mice. Our data indicates that the glycan part of zymosan (β-glucan and mannan) may be involved in this process by their modification of the TLR-triggered DC phenotype.

All β-glucans were found to down-regulate the expression levels of Dectin-1, and also to varying degrees the levels of MR and DC-SIGN, dependent on the β-glucan preparation in question, thus suggesting that some components in the β-glucan-containing products interact with these CLRs. It is well documented that β-glucans bind to Dectin-1 [233], whereas MR and DC-SIGN recognize oligo-mannose and fucose residues [24, 25]. The suppression of MR and DC-SIGN expression levels by lichenan, zymosan and also yeast β-glucan therefore points to the presence of mannose or fucose-containing glycans in these preparations. By analyzing the polysaccharide composition of these products, we detected mannose in yeast β-glucan and lichenan, and in zymosan, consistent with earlier reports [193]. The presence of mannans in some β-glucan products may well interfere with the interpretation of the effect of β-glucans on modification of TLR-triggered DCs, and likewise for other yet un-identified CLR-ligands that may be present in the products. In our designation of a general β-glucan-induced DC phenotype, we have therefore taking this uncertainty point into consideration, and included only those proteins that were regulated identically by all β-glucan products.

Our data on CLR regulation by the diverse β-glucans products demonstrates that Dectin-1, MR and DC-SIGN are regulated in a ligand-receptor specific manner, also upon TLR-induced activation. Activation of CLRs by β-glucans may be an important contributor to the phenotypic profile in TLR-triggered DCs, as CLR-mediated modification of TLR-induced profiles in DCs is acknowledge as a regulatory paths playing a major role for pathogenic escape mechanisms [201, 234]. Based on our current data, we are not able to deduce whether it is Dectin-1, DC-SIGN, MR, the interplay between the 3 receptor pathways or yet other CLRs that mediate the β-glucan-induced modulation of the TLR-triggered phenotype in DCs. Moreover, we still lack significant insight into how and at what level the CLR and TLR signaling pathways interact, and which TLRs that are affected by which CLRs. Although we did not address the exact role of individual CLRs in shaping the β-glucan-induced DC phenotype in this study, we consider it likely that CLRs, such as dectin-1, are involved in the β-glucan-mediated modification of the TLR-induced activation signal in DC.

Collectively, we here identified a pluripotent β-glucan-modified phenotype in TLR-triggered DC. Two functional DC subsets were found that may explain the overall fingerprint including simultaneous secretion of Treg and Th17-inducing cytokines and chemokines in the β-glucan- and TLR-primed DCs. This general feature of β-glucan-mediated changes in the TLR-induced DC phenotype may be of vital importance for the direction of adaptive immune responses against fungal infections.

CHAPTER 5

MODIFICATION OF *IN VIVO* ANTIGEN-SPECIFIC IMMUNE RESPONSES BY A RECOMBINANT FUSION VARIANT OF AN ANTIGEN AND A β-GLUCAN BINDING MODULE: ADJUVANT PROPERTIES OF LICHENAN

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Lichenan is, like many other β-glucans, a potent immune modulator and may find application as an adjuvant agent. To increase insight into *in vivo* modulatory properties of lichenan, we examined the effect of lichenan on the specific immune response against a protein injected into mice with focus on the importance of complex formation between protein and lichenan for the immunomodulating effect. To study the influence of association between protein and the β-glucan, a fusion protein, consisting of porcine β-lactoglobulin and a carbohydrate binding domain that binds non-covalently to β-glucan, was produced. By use of the fusion protein, the *in vivo* immunomodulatory effect of lichenan was studied and compared to the *in vitro* response against lichenan in murine dendritic cells. The β-glucan-binding fusion protein or β-lactoglobulin alone was mixed with lichenan, lipopolysaccharide or both, and injected into mice. β-lactoglobulin-specific antibody titers revealed that lichenan caused a significant increase in the antibody response, but only if lichenan was combined with the fusion protein. This was caused by an increase in β-lactoglobulin-specific IgG1 and IgG2a antibodies, as in contrast to co-injection of lichenan with β-lactoglobulin resulting in an IgG1 response only. When lichenan and the fusion protein were co-injected with lipopolysaccharide, further enhancement of the lipopolysaccharide-induced β-lactoglobulin-specific IgG2a response was observed. These *in vivo* observations are in contrast to the modulatory properties of lichenan in LPS-activated antigen presenting cells as observed *in* vitro. In conclusion, lichenan improves the Th1-adjuvant properties of lipopolysaccharide, and exhibits antigen-specific Th1/Th2-polarizing adjuvant activity when non-covalently associated to antigen.

INTRODUCTION

β-glucans from fungi, yeast and plants have shown to possess immune modulatory ability [235]. *In vitro* studies have demonstrated immune regulatory effects on cells of both the innate and the adaptive immune system, especially in antigen presenting cells (APC), such as dendritic cells (DC) and macrophages (mϕ) where modulation of cytokine expression levels and phagocytosis is reported [120-122, 126, 127]. From *in vivo* investigations, β-glucans are reported to hold immunostimulatory activities, and may accordingly influence pathogenic clearance [147] and possess adjuvant properties [236, 237]. In particular, fungal (1,3)-β-glucan are observed to increase the antigen-specific immunoglobulin (Ig) response, especially the isotype IgG1, when injected subcutaneously with ovalbumin [236, 237]. IgG subtype responses induced upon immunization are indirect measures of the relative contribution of Th2-type cytokines *versus* Th1-type cytokines. Th2-type cytokines promoting the production of IgG1-type antibodies, while IgG2a-type antibodies reflect the involvement of Th1-type cytokines [238].

Not only immunostimulating properties of β-glucans have been demonstrated. In a few studies, β-glucans were also shown to reduce the pro-inflammatory cytokine responses induced by other microbial components. We and others [207, 239] have found that co-stimulation of DCs with β-glucans and Toll-like receptor (TLR) ligands results in enhanced IL-10 and TNF-α levels while decreasing IL-12p70 production as compared to TLR-induced responses alone.

In general, when comparing β-glucans of microbial origin with those from plants, the microbial β-glucans have demonstrated strongest immunomodulating activities [207]. Microbial and algae β-glucans consist of alternating sequences of (1,3) and (1,6)-β-glucosidic linkages, whereas plant β-glucans in general are (1,3),(1,4)-β-glucan sequences containing short blocks of (1,4)-β-linked glucosyl residues connected by (1,3)-β-linkages [191, 199].

Lichenan, a mixed linkage (1,3),(1,4)-β-glucan from the lichen *Cetraria islandica*, has been demonstrated to possess immune modulating effects [96]. *In vitro,* lichenan induced high levels of IL-10 and low levels of IL-12 in DCs [96, 104], and in an arthritis model in mice, lichenan reduced inflammation, probably due to a bias in cytokine production from IL-12 towards IL-10 production [240]. Whether lichenan holds adjuvant activity or other immunomodulating activities in relation to the adaptive immune response has to our knowledge not been addressed.

APC, especially DC, are key players in initiation of adaptive immune responses as they take up antigens and through their display of various pattern recognition receptors (PRRs) become activated by interacting with a variety of stimulating agents. PRRs comprise different groups of receptors, including the TLRs and the C-type lectin receptors (CLRs) [28]. Depending on the nature of the stimulating compound, the DCs will orchestrate primarily a cell-mediated, an antibody-mediated, or a regulatory type immune response towards the phagocytosed antigen. The recognition of (1,3)-β-glucans by DCs is mediated by members of the CLR PRR family, especially dectin-1[28, 77]. CLRs contribute to internalization of antigen upon carbohydrate-mediated interactions and binding, resulting in maturation of the DCs via cross-talk with TLR signaling pathways [241]. The binding of lichenan and other mixed linkage (1,3),(1,4)-β-glucans to dectin-1 has been demonstrated in DCs [233], and our own unpublished data show that lichenan enhances the production of the cytokines IL-10 and IL-2 in DCs in a TLR-dependent manner. Whether CLR-mediated antigen uptake depends on a tight association between the CLR-ligand and the protein antigen to be presented by MHC class II on the APCs has not been thoroughly studied. By targeting CLRs with ligands, it might be possible to enhance antigen presentation or to direct activation of specific T cell subsets. Such applications are useful for immunotherapy purposes or in vaccine design. Experiments that target CLRs with specific antibody-conjugated proteins have shown an increased antigen uptake in DCs, but only slight activation of signaling pathways [173]. Targeting of CLRs through direct association between protein and glucan molecules may, however, act differently and induce other regulatory pathways. This was previously demonstrated by Xie *et al*., by use of the low Mw β-glucan ligand laminarin covalently conjugated to ovalbumin resulting in enhancement of the ovalbumin-specific immune response [242]. Moreover, a conjugate of bovine serum albumin and microparticulate β-glucan from yeast was demonstrated to generate an antigen-specific antibody response after dermal injection [243]. Both studies reported an increase in the antigen-specific antibody response in presence of β-glucans and pointed towards an efficient immune activation by β-glucans, independently of the size of the β-glucan ligand. As (1,3),(1,6)-β-glucans were applied in both studies it is not known whether (1,3),(1,4)-β-glucans, e.g. lichenan, exhibit the same immunostimulating properties. Furthermore, it is of interest to know how tight the antigen needs to be associated to the CLR-ligand to obtain the adjuvant effect. A covalent conjugation is possibly not a strict requirement, if a specific affinity driven binding between ligand and antigen exists.

In the present study, we examined the potential of the β-glucan lichenan to engage as an adjuvant and induce an antigen-specific response towards an antigen when mixed and co-injected together, as compared to being non-covalently associated to the antigen in a fusion protein consisting of the antigen, porcine β-lactoglobulin (BLG) and a β-glucan-binding domain (CBM) from *Clostridium thermocellum*. In addition, we studied if association of the antigen to PRRs could modulate the immune response against the antigen when co-injected with the TLR-4 ligand lipopolysaccharide (LPS) to trigger Th1-polarizing immune responses *in vivo*.

MATERIALS AND METHODS

Animals

BALB/c mice, 8 weeks old were from Taconic Europe, Lille Skensved, Denmark.

All animals' studies were approved by The Danish Animal Experiments Inspectorate and were carried out according to the guidelines of "The Council of Europe Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific purpose". Permission number: 2007/561-1266.

Strain, Plasmids and production of amplicons and constructs

Escherichia coli vector pET21a(+) (Novagen, San Diego, CA), *Pichia pastoris* pPICZαA (Invitrogen, Carlsbad, CA), *E. coli* TOP10 competent cells (Invitrogen), *E. coli* BL21 (DE3) (Novagen) and *P. pastoris* X-33 (Invitrogen) were used for cloning and heterologous expression of the constructs: *Ct*CBM11 [244], BLG [245], and the fusion protein (*Ct*CBM11_BLG; CBM-BLG) (Fig. 1). The *E. coli* expression vector encoding the family 11 carbohydrate binding module (*Ct*CBM11) [244] from the bi-functional cellulosomal cellulase-lichenase from *Clostridium thermocellum* (*Ct*Lic26A-Cel5E) was a kind gift of Professor Harry J. Gilbert (Institute for Cell and Molecular Biosciences, University of Newcastle upon Tyne). The *P. pastoris* expression vector encoding the BLG [245] was kindly provided by Professor Marina Lotti (Dipartimento di Biotechnologie e Bioscience, Università degli Studi di Milano-Bicocca).

Plasmid DNA of *Ct*CBM11 (GenBank number AAA23225.1, amino acids 655-821) or porcine BLG (GenBank number AAQ74978.1, amino acids 19-178) was used as a PCR amplification template for generating the isolated BLG and the fusion protein, which was obtained by using

overlap PCR. The primers used for amplification was BLG sense primer (5′-CCG **CTC GAG** AAA AGA GAG GCT GAA GCT GTT GAA GTT ACC CCA ATT ATG-3′), BLG antisense primer (5′-G**CT CTA GAT** CAA TGA TGA TGA TGA TGA TGG ACG CAC TGC TCT TC-3′), *Ct*CBM11 with BLG overlap sense primer (5′-GAA GAG CAG TGC CGC GTC GCT GTC GGT GAA AAA ATG -3′), BLG with *Ct*CBM11 overlap antisense primer (5′- GCA TTT TTT CAC CGA CAG CGA CGC GGC ACT GCT CTT C-3′) and *Ct*CBM11 sense primer (5′- CCG **CTC GAG** AAA AGA GAG GCT GAA GCT GCT GTC GGT GAA AAA ATG-3′). *Ct*CBM11 sense primer (5′-**GCT AGC** TAG CGC TGT CGG TGA AAA AAT GCT GGA TG-3′) and *Ct*CBM11 antisense primer (5′-CCG **CTC GAG** GAC GCG GCA CTG CTC TTC -3′).

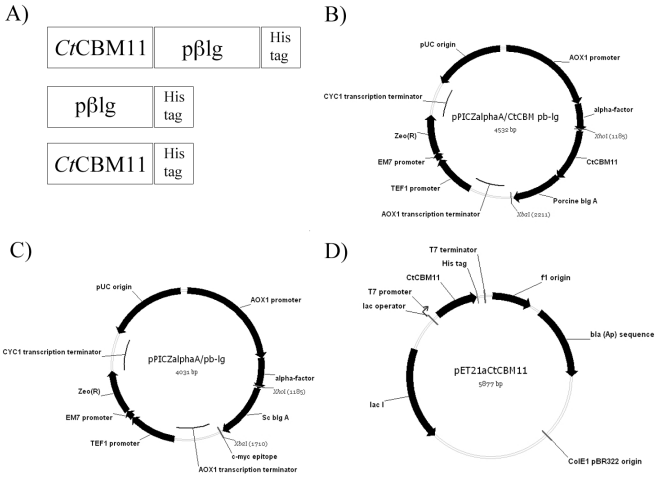


Figure 1. (A) Schematic representation of the different PCR products. Plasmids transformed into the production strains. Plasmids pPICZαA containing either the CBM-BLG (B) or BLG (C) for transformation into the production strain *P. pastoris* X-33. (D) Plasmid pET21a(+) containing *Ct*CBM11 for transformation into *E. coli* BL21 (DE3).

The PCR products BLG and the fusion protein CBM-BLG (Fig. 1) were digested with restriction enzymes (Xho1, Xba1 and Nhe1) and cloned into the *E. coli* expression vector pET21a (+) or the *P. pastoris* pPICZαA. Products encoded by these vectors contain a C-terminal His_6 tag. The different plasmids were propagated in *E. coli* TOP10 maintenance strain (Invitrogen), purified (QIAprep Spin Miniprep kit, Qiagen, Germantown, Germany) and sequenced before transformation into expression strains: *E. coli* BL21 (DE3) (Novagen) or *P. pastoris* X-33 (Invitrogen). The *P. pastoris* expression vector was linearized with Pme1 before transformation into *P. pastoris* by electroporation according to the manufacturer's recommendations. Transformants were selected on YPDS plates (1% w/v yeast extract, 2% w/v peptone, 2% w/v glucose, 1 M sorbitol, 2% w/v agar and 100 μg/ml Zeocin) (Invitrogen).

Protein production

CBM: A single colony was inoculated into 50 mL LB media supplemented with antibiotic amplicilin (100 μg/mL), grown overnight at 30 ^{o}C, used to inoculate 1L LB supplemented with the amplicilin (100 μg/mL) to a starting OD_{600} = 0.05-0.1, and grown at 30 ^{o}C until $OD_{600} \approx 0.5$. The culture was induced with isopropyl β-D-thiogalactopyranoside (0.5 mM) (Sigma Aldrich, St. Louis, MO) for 4 h. The cells were harvested by centrifugation and lysed with Bugbuster (Novagen) (10 mL/L) followed by addtion of 2 mM $CaCl_2$

BLG and CBM-BLG: A single colony was inoculated into 25 mL BMGY-media (1% w/v yeast extract, 2% w/v peptone, 0.1 M potassium phosphate pH 6.0, 1.34% w/v Yeast nitrogen base with ammonium sulfate without amino acids, $4x10^{-5}$% w/v biotin and 1% w/v glycerol) at 30 ^{o}C until OD_{600} = 2-6, then was the cells harvested and the cells were resuspended in 0.4 L BMGY. The culture was incubated at 25 ^{o}C with vigorous shaking until OD_{600} = 2. The cells were harvested by centrifugation and induced for 48 h by changing the medium to 0.4 L BMMY (1% w/v yeast extract, 2% w/v peptone, 0.1 M potassium phosphate pH 6.0, 1.34% w/v yeast nitrogen base with ammonium sulfate without amino acids, $4x10^{-5}$% w/v biotin and 0.5% w/v glycerol). Methanol was added to 0.5% (v/v) every 24 h. After 48 h the culture was centrifuged and the supernatant was collected. EDTA-free Complete Protease inhibitor cocktail (Hoffmann-La Roche AG, Basel, Switzerland) and 2 mM $CaCl_2$ was added and the pH was adjusted to 7.0 before filtration on 0.45 μm membrane filter.

Protein purification

The recombinant CBM, BLG and CBM-BLG were purified by immobilized metal ion affinity chromatography on a 1 ml His-trap column (GE Healthcare, Uppsala, Sweden) equilibrated with 10 mM HEPES, 0.5 M NaCl, 20 mM imidazole, pH 7.5 and eluted using a linear gradient (0 –100 % over 20 column volumes) of this buffer with 10 mM HEPES, 0.5 M NaCl, 400 mM Imidazole, pH 7.5.

Surface Plasmon Resonance

The proteins (30 μg/mL) were immobilized on a CM5 chip (GE Healthcare) at pH 4 using the random amine coupling kit (GE Healthcare). Surface plasmon resonance (SPR) analysis was carried

out using a Biacore T100 (GE Healthcare, Uppsala, Sweden) and sensorgrams were recorded for the binding of 1–1000 nM barley β-glucan (Mw = 20 kDa, Novozymes, Bagsværd, Denmark) to CBM or CBM-BLG, respectively. Apparent dissociation constants (K_d) values were calculated using a steady state affinity model in Graphpad Prism 5.03 (GraphPad software, La Jolla, CA). The steady state response data were fitted the model after subtracting the reference cell signal and normalizing with the response for buffer injections.

Binding of insoluble polysaccharides

CBM from *C. thermocellum* shows affinity for β-(1,4)- and β-(1,3),(1,4)-mixed linked glucans [244]. Qualitative assessment of the fusion proteins binding to lichenan (Megazyme, Wicklow, Ireland) (Mw = 94 kDa) was carried out as described by Carvalho *et al*. [244] with minor modification. In brief, lichenan was suspended in water and the insoluble part was spun down and then dried. 1 mg was mixed with 30 μg of protein in 50 mM Tris-HCl buffer, pH 7.5, containing 0.05% (v/v) Tween 20 and 5 mM $CaCl_2$ (Buffer A) in a reaction volume of 200 μL. The mixture was incubated for 2 h at 4 °C with gentle shaking and afterwards the insoluble fraction was pelleted by centrifugation (13000 x *g*, 5 min). The supernatant, containing unbound protein, was removed, and the pellet was washed three times with 200 μl buffer A. The bound protein was eluted by boiling the pellet in 150 μL buffer A plus 50 μL Nupage LDS sample buffer (Invitrogen) containing Nupage sample reducing agent (Invitrogen) for 10 min. Bound and unbound protein fractions were analyzed on a Nupage 4–12 % Bis-Tris gel (Invitrogen). Controls containing BLG or CBM-BLG without β-glucan co-incubation were performed in parallel.

Immunization protocol

Mice were immunized intraperitoneally (i.p.) at days 1 and 14. Blood was collected from the retro-orbital plexus into heparin-coated capillaries on days 1 and 21, and immediately mixed with phosphate buffered saline (0.01 M, pH 7.4) containing 0.05 % Tween 20 (PBS-T) (1:16) and stored at -20^{o}C.

Mice divided into 8 groups were immunized with mixtures containing LPS (1 μg), BLG (20 μg), CBM-BLG (40 μg) and lichenan (50 μg) in LPS-free saline solution, distributed as depicted in table 1. The different reaction mixtures were incubated for 2 h at 4 °C with gentle shaking, without LPS. Right before injection LPS were added and mixed. Lichenan was before addition to the mixture

suspended in water and the insoluble fraction was used. The injected volume was 200 μL/mouse. The dose of co-administered LPS required boosting the BLG-specific IgG production was tested in a pre-study (data not shown). This study was also used to determine the group sizes for the final experiment. The initial experiment was similar to the final experiment, but with fewer mice.

Table 1: Immunization solution content for the different groups

		LPS	BLG	CBM-BLG	Lichenan
Group nr:					
-	1			X	
-	2			X	X
-	3	X		X	
-	4	X		X	X
-	5		X		
-	6		X		X
-	7	X	X		
-	8	X	X		X

Determination of specific antibody by ELISA

BLG (10 μg/mL) in sodium carbonate buffer (0.05 M, pH 9.6) was coated onto microtiter plates (Maxisorp, Nunc, Roskilde, Denmark) by overnight incubation at 4°C. After washing of plates, blood samples were added and serially two-fold diluted in PBS-T. The plates were incubated for 1 h at room temperature, washed and incubated with peroxidase-conjugated anti-mouse immunoglobulin (Ig) (Dako, Glostrup, Denmark) or anti-IgG1/IgG2a (AbD Serotec, Raleigh, NC). Enzyme-catalysed color development was accomplished by adding TMB (Merck, Darmstadt, Germany), diluted in peroxide solution, for 10 min and stopped with phosphorus acid (2 M). Plates were read at 450 nm using 630 nm as reference. On each plate an internal standard (pooled blood) was included. Results were expressed as $\log_2$ titers defined as the sample dilution giving an absorbance of 0.2.

Generation of DC and analysis of surface molecule expression and cytokine production

Bone marrow (BM) cells were isolated from C57BL/6 mice (Taconic Europe, Denmark) as described previously [185]. To cultivate DCs, 10 mL cell suspension containing $3x10^6$ stem cells was seeded in 100-mm bacteriological petri dishes at day 0 (Greiner bio-one, Kremsmünster, Austria) and incubated for 8 days at 37 °C and 5% CO_2. On day 3, an additional 10 mL cell culture medium (RPMI 1640 added 10% heat inactivated FBS, 2 mM L-glutamin, 50 μM 2-mercaptoethanol 100 units/ml penicillin, 50 μg/ml streptomycin and 15 ng/ml GM-CSF) was added.

At day 6, cell culture medium was replaced by fresh medium. On day 8, the non-adherent cells were gently pipetted from the petri dishes and centrifuged for 5 min at 280 g. The cells were resuspended in fresh cell culture medium without GM-CSF, and seeded in 48-well culture plates (Corning inc., Corning, NY) at $1x10^6$ cells/600 μL well. DCs were cultured with lichenan with or without LPS (1 μg/ml, *E. coli* O26:B6; Sigma-Aldrich Inc.). Cells added medium alone were used as untreated DCs. After stimulation for 18 h, culture supernatants were collected and stored at -20 ^oC until cytokine analysis. The cytokines IL-6, IL-10, IL-12p70 and TNF-α were analyzed using commercially available ELISA kits (R&D systems, Minneapolis, MN) according to the manufacturer's instruction. Detection limits for the different cytokines were; IL-6: 5 pg/mL; IL-10: 10 pg/mL; IL-12p70: 4 pg/mL; TNF-α: 8 pg/mL.

To test for surface molecule expression, DCs were generated and stimulated as described above except for seeding in 12-well culture plates (Nunc, Roskilde, Denmark) at day 0. Upon stimulation at day 8, cells were treated with ice cold PBS-az; containing 1% (v/v) heat-inactivated FBS and 1.5% (w/v) sodium azide (Sigma-Aldrich Inc.) to prevent internalization of surface markers during subsequent handling of the cells. DCs were thereafter kept at 4^oC or below. To block non-specific binding of Abs, cells were incubated with anti-mouse FcγIII/II receptor antibody, clone 2.4G2 (3 μg/ml, BD Bioscience, San Jose, CA) before addition of fluorochrome-conjugated Ab. Upon incubation, cells were washed twice in PBS-az before analysis on a BD FACSCanto II (BD Bioscience). The analysis was based on 20,000 cells and gated on viable cells. The Abs used were: APC-conjugated anti-mouse CD11c, clone N418, PE-conjugated anti-mouse CD40, clone 1C10, PE-conjugated anti-mouse CD80, clone 16-10A1, PE-conjugated anti-mouse MHCII, clone NIMR-4, all eBioscience, San Diego, CA. APC-conjugated anti-mouse CD86, clone GL1 (Southern Biotech, Birmingham, AL). Isotype-matched controls: APC-conjugated Rat IgG2a, clone R35-95 (BD Bioscience), PE-conjugated rat IgG2b, clone KLH/G2b-1-2 (Southern Biotech), PE-conjugated Armenian hamster IgG, clone eBio299Arm (eBioscience), PE-conjugated Rat IgG2a (eBioscience).

Data were analyzed using FCS Express (version 3.0, De Novo Software, Los Angeles, CA).

Statistical analysis

The data were analyzed for statistical significance (GraphPad Prism, version 5.01) using one-way ANOVA and the Tukey post test. A P-value < 0.05 was considered significant (*), P < 0.01 = ** and P < 0.001 = ***.

RESULTS

Lichenan modulates the phenotype of dendritic cells.

To increase our understanding of the immunoregulatory effects of the plant-derived β-(1,3),(1,4)-glucan lichenan, we first examined the modulation of DCs by lichenan and looked into the regulation of molecules of importance for the T helper cell polarization; MHC class II, CD40, CD80, CD86, IL-10 and IL-12p70. Lichenan *per se* was observed to induce up-regulation of MHCII, CD86, but not CD40 and CD80 in DCs (Fig. 2A, B). IL-10 and IL-12p70 production from DCs was noticed not to be affected (Fig. 2C). In comparison, LPS stimulated a much stronger induction of CD40, CD86, IL-10 and IL-12p70 than lichenan, whereas levels of MHC class II and CD86 were similar (Fig. 2A, B). When lichenan and LPS were added to DCs simultaneously, lichenan affected the LPS stimulatory effect on DCs by down-regulating LPS-induced IL-12p70, CD40 and CD80 while, at the same time, up-regulating IL-10 and CD86, and also slightly the MHC class II levels (Fig. 2). Thus, in these *in vitro* studies, we observed that lichenan holds immunoregulatory properties that collectively promote suppression of molecules in DCs that are important for Th1-generation. Due to the potency of lichenan to regulate the DC phenotype *in vitro*, we found it of interest to examine the *in vivo* immunomodulatory capacities of lichenan and focused on studying modulation of antigen-specific antibody responses, which is of importance for vaccine adjuvants. In order to investigate whether, i) lichenan is able to act as an adjuvant and initiate a specific immune response against a protein antigen, and ii) if a tight association between the antigen and lichenan would be a prerequisite for this immune modulatory effect, a recombinant fusion protein featuring a carbohydrate-binding module (*Ct*CBM11) fused to the protein BLG, was constructed. The CBM module exhibits affinity in the low μM range for (1,3),(1,4)-mixed linked glucans.

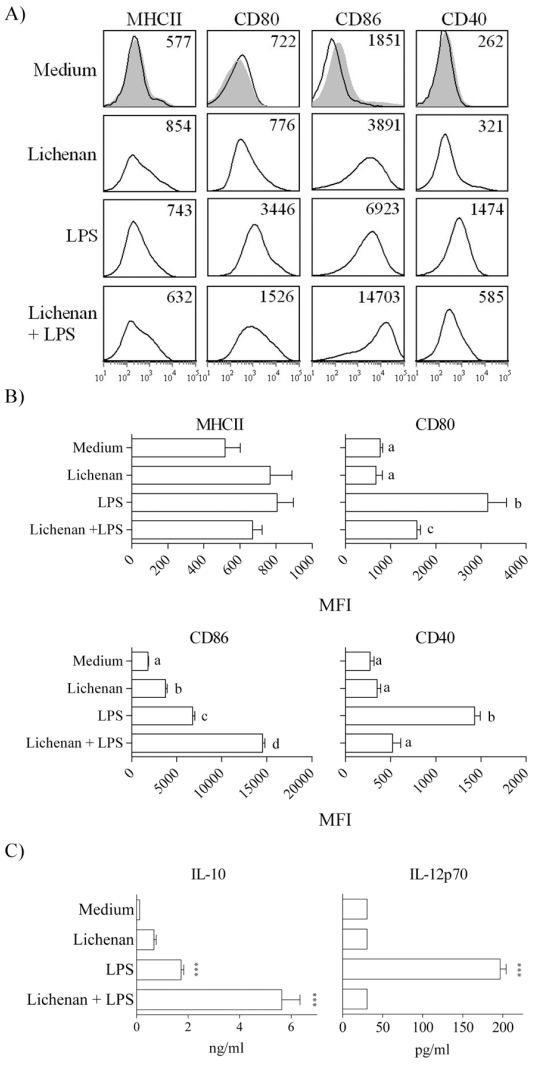


Figure 2. Influence of lichenan on the phenotype of dendritic cells.
Murine bone marrow-derived DC ($1 \cdot 10^6$ cells/0.6 ml/well) were incubated for 18 h with LPS (1 μg/ml), lichenan (200 μg/ml), lichenan + LPS or medium (immature DC). (A) Expression of MHC II and the co-stimulatory molecules CD80, CD86 and CD40 in response to lichenan, LPS alone and lichenan together with LPS. The cells were stained for surface markers indicative of maturation. Numbers represent the mean fluorescence intensity of the marked area. The dotted line represents cells stained with isotype control antibodies. (B) Comparision of data from two independent experiments described in A. Bars labelled with the same letter (a, b or c) indicate no significant difference (P>0.05). Data are mean ± SD (n=2). (C*)* Levels of IL-10 and IL-12p70 in DC supernatants upon 18 h of incubation as measured by ELISA, Data are mean ± SD (n=3).

Production and characterization of a β-glucan-binding fusion protein.

The recombinant fusion protein (CBM-BLG) was constructed via overlap PCR and expressed in *P. Pastoris* X-33. CBM11 was expressed in *E. coli* BL21 (DE3) and BLG in *P. pastoris* X-33. SDS-PAGE analysis confirmed the molecular size of recombinant CBM-BLG and BLG as roughly 37 and 18 kDa, respectively (Fig. 3C, lane 1 and 4).

The β-glucan binding ability of CBM-BLG was established, with CBM as the reference, in a surface plasmon resonance (SPR) setup using a low Mw barley β-glucan as analyte. Based on these analyses, K_d values of $2x10^{-6}$ M for the binding of CBM-BLG to the β-glucan and $2x10^{-5}$ M for the CBM were determined (Fig. 3A, B).

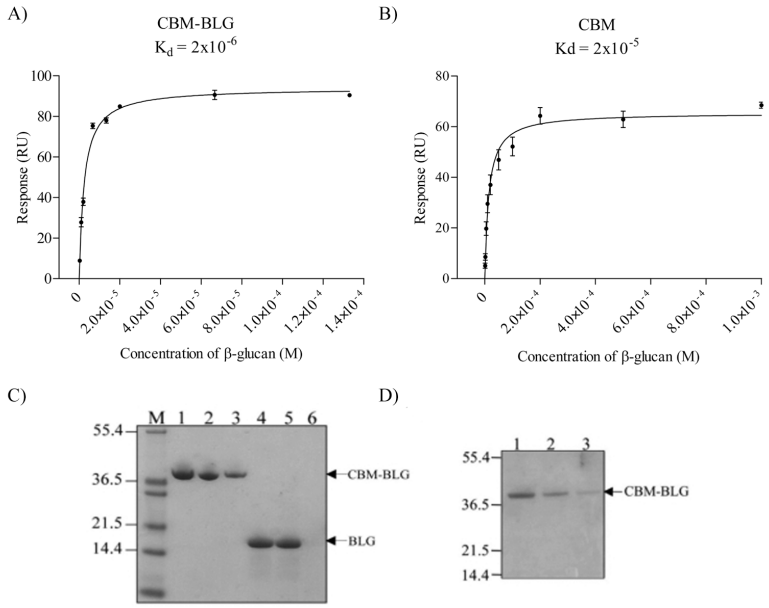


Figure 3. Characterization of a β-glucan-binding fusion protein.
CBM in CBM-BLG mediates binding of β-glucans. Apparent binding affinity of the CBM-BLG (A) and CBM alone (B) towards a barley (1,3),(1,4)-β-glucan is demonstrated experimentally with sensorgrams recorded upon injecting barley β-glucan on sensorchips immobilized with CBM-BLG. The Langmuir binding isotherm for one binding site was fitted to the Surface Plasmon Resonance response data to calculate the apparent dissociation constant. (C) Mw of CBM-BLG (*lane 1-3*) and BLG (*lane 4-6*) as assessed by SDS-PAGE**.** The binding affinity of CBM-BLG to lichenan was tested in an adsorption assay. Protein was mixed with insoluble lichenan and incubated for 2 h at 4 °C. Lichenan was pelleted and the supernatant was collected. *Lane 2 and 5* show the amount of protein in the supernatant. The pellet was washed three times. Protein bound to insoluble lichenan was eluted by boiling in 10 % (w/v) SDS, *lane 3 and 6* shows the amount of protein precipitated with lichenan. The MW marker is displayed in *lane M*. (D) Supernatant from wash 1, 2 and 3 of the pellet containing lichenan and CBM-BLG.

The binding of CBM-BLG to the insoluble residue of lichenan was demonstrated in an adsorption assay, as the use of SPR was precluded due to the size and poor solubility of lichenan in aqueous solutions. The binding assay relies on the adsorption of CBM-BLG to the insoluble lichenan and thereafter the CBM-BLG/lichenan complex can be recovered in the pellet by centrifugation of the suspension. Upon incubation of lichenan with CBM-BLG, SDS-PAGE of both supernatant and the pellet confirmed that a substantial fraction of CBM-BLG remained bound to lichenan after three consecutive washes (Fig. 3C, lane 1-3), while BLG, when present alone, did not bind to lichenan (Fig. 3C, lane 4-6). SDS-PAGE of the supernatant from repeated washes of the pellet (prior to SDS-PAGE of pellet in Fig. 3C, lane 3) demonstrated that some CBM-BLG was released from the pellet while washing with an aqueous solution (Fig. 3D, lane 1-3), but that detectable release of CBM-

BLG drops steeply upon three washes. The remaining CBM-BLG in Fig. 3C, lane 3 was therefore tightly associated with the precipitated lichenan.

Lichenan non-covalently associated to BLG acts as an adjuvant

To test whether lichenan could act as adjuvant and thus enhance the specific antibody response towards BLG, and if a non-covalent attachment of BLG to lichenan would further enhance the antibody response, mice were immunized with either of the two different antigens (BLG or CBM-BLG), or with a mixture of one of the antigens and the insoluble fraction of lichenan. The mixtures were incubated for two hours and centrifuged prior to immunization. Moreover, to test whether lichenan could modulate the BLG-specific antibody response induced, the same samples were injected after mixing with LPS. Immunization with antigens and LPS was used as controls. Before the first immunization, blood samples from all mice were tested for response towards BLG (antibody IgG titer average of 7.0 ± 0.3).

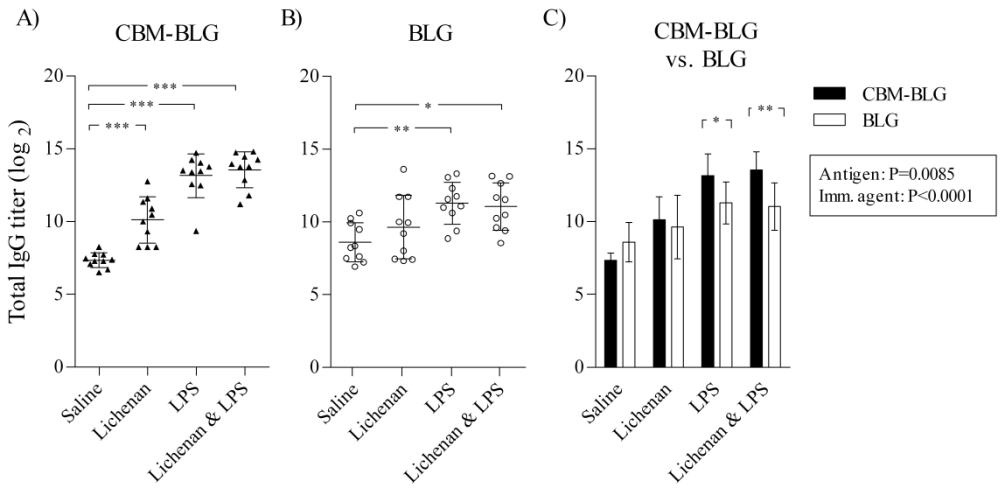


Figure 4. IgG antibody response against an antigen complex-bound to lichenan.
BLG-specific IgG production in mice immunized with lichenan mixed together with CBM-BLG or BLG. Mice were immunized (i.p.) on day 1 and 14 and tested for plasma titers of BLG-specific IgG in blood samples drawn one weekend after last immunization. Antibody titers are represented as the $\log_2$ of the reciprocal of the sample dilution giving an absorbance of 0.2. IgG response from mice immunized with CBM-BLG (A) or BLG (B) alone (saline), together with lichenan, LPS or lichenan + LPS. (C) Comparison of the response against CBM-BLG and BLG when injected alone (Saline), or with lichenan, LPS or lichenan + LPS. Data are mean ± SD (n=10). *, $p<0.05$, **, $p<0.01$, ***, $P < 0.001$ as tested by: (A) + (B) one-way ANOVA with Tukey posttest, (C) two-way ANOVA with Bonferroni posttest.

LPS mixed with either the CBM-BLG or BLG resulted in a significantly higher BLG-specific IgG antibody response than the mice injected with the CBM-BLG or BLG in PBS alone (Fig. 4A, B), thus demonstrating the adjuvant effect of LPS. Immunization with lichenan in a non-covalent complex with CBM-BLG resulted in a significant increase in BLG-specific IgG antibodies, whereas

immunization with lichenan mixed with BLG resulted only in a modest, but insignificant increase (Fig. 4A, B), implying that the association between lichenan and the antigen may enhance the immune response against the antigen. When injecting LPS together with the mix of lichenan/antigens, no further boost in the total BLG-specific IgG response pattern was seen, as compared to the effect of LPS and antigen alone (Fig. 4A, B). A comparison of the BLG-specific IgG response induced against the CBM-BLG versus the BLG alone (Fig. 4C) revealed a significant difference in BLG-specific IgG responses between CBM-BLG and BLG (P=0.0085), upon immunization together with LPS with or without lichenan.

To examine the contribution of specific IgG isotypes on the total IgG profile, we measured the levels of BLG-specific IgG1 (Th2) and IgG2a (Th1) antibodies from the immunized mice.

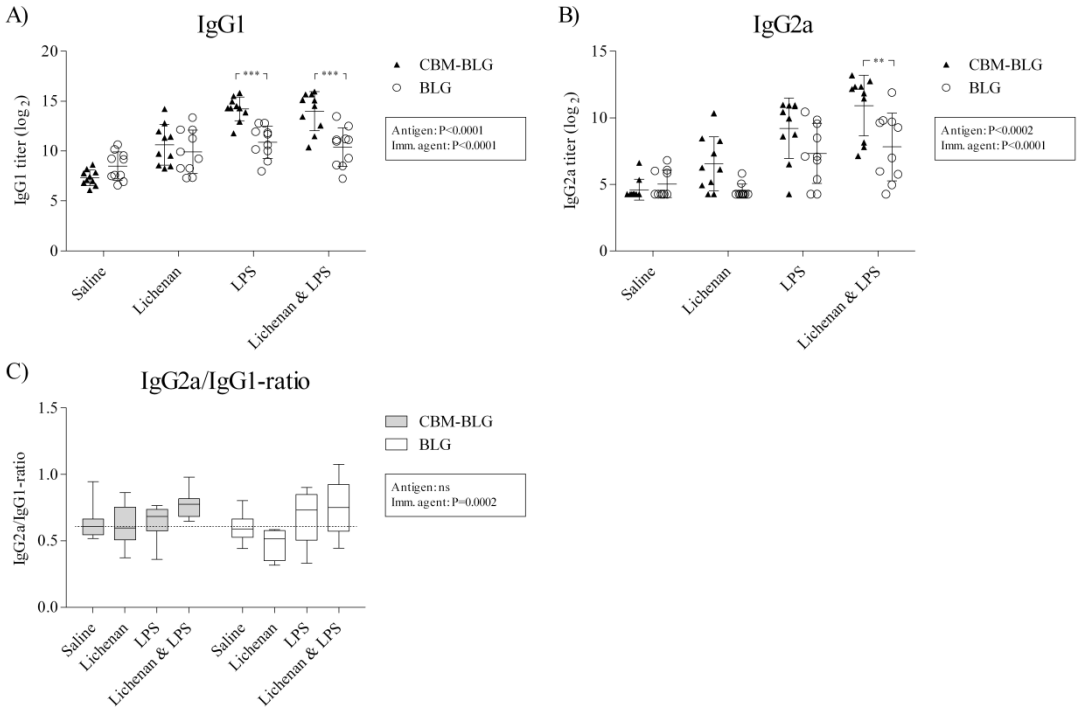


Figure 5. Th1/Th2 polarizing properties of lichenan.
BLG-specific IgG1 and IgG2a production in mice immunized with lichenan mixed together with CBM-BLG or BLG. Mice were immunized (i.p.) on day 1 and 14 and tested for plasma titers of BLG-specific IgG1 and IgG2a in blood samples drawn one weekend after last immunization. Antibody titers are represented as the $\log_2$ of the reciprocal of the sample dilution giving an absorbance of 0.2. IgG1 (A) or IgG2a (B) response from mice immunized with CBM-BLG or BLG alone (saline), together with lichenan, LPS or lichenan + LPS. (C) Comparison of the response against CBM-BLG and BLG when injected alone (saline), or with lichenan, LPS or lichenan + LPS as a ratio between IgG2a and IgG1. Data are mean ± SD (n=10). *, p<0.05, **, p<0.01, ***, P < 0.001 as tested by a two-way ANOVA with Bonferroni posttest.

Generally, we observed a similar response pattern for BLG-specific IgG1 levels as seen for total IgG (Fig. 5A), which is common in BALB/c mice, whereas levels of BLG-specific IgG2a were only

induced by specific agents; *i.e.* lichenan mixed with CBM-BLG, and both antigens when co-injected with LPS with or without lichenan (Fig. 5B). To describe patterns of Th1/Th2-polarizing properties by the antigens in the context of lichenan, LPS or both, we compared the ratios of BLG-specific IgG2a/IgG1-antibodies (Fig. 5C). This comparison showed a higher tendency to IgG2a production when co-injecting both antigens with LPS with or without lichenan, and with lichenan and CBM-BLG giving rise to a significant increase in IgG2a antibodies. This was in contrast to the effect of co-administration of lichenan with BLG, thus suggesting a Th1-polarizing potential of lichenan when associated with the antigen.

DISCUSSION

In the present work, the adjuvant effect of lichenan on the specific immune response towards a protein antigen was demonstrated as lichenan possessed the capacity to function as an adjuvant, but only to a significant degree if the antigen was directly associated with lichenan. Although lichenan has previously been found to hold immunomodulatory effects, this is the first time lichenan was shown to possess adjuvant properties.

The fusion protein CBM-BLG composed of porcine BLG and the carbohydrate binding module of *Clostridium thermocellum* Lic26A Cel5E [244], expressed in *P. pastoris,* was incubated with the insoluble fraction of lichenan. By i.p. injection of this mixture, we were able to induce a significantly higher BLG-specific antibody response than when the BLG was injected *per se.* The response was further enhanced when the same protein was injected with LPS. This demonstrates that β-(1,3),(1,4)-glucans may hold adjuvant properties and that non-covalent association of the antigen to the β-glucan is sufficient to confer this adjuvant activity.

It has earlier been shown that a tight association between antigen and β-glucan is required for an adjuvant effect of β-glucans [242, 243]. In those studies, the protein antigen was covalently bound to the β-glucan. Here we show for the first time that non-covalent association is sufficient for obtaining the adjuvant effect and suggest that this property may have implications for diverse food matrices consisting of food antigens and polysaccharides, as well as for formulation of new adjuvants and vaccines. Although it was not possible to determine the precise binding affinity of the CBM-BLG to the high Mw lichenan, the CBM-BLG was shown to form a stable association with the insoluble fraction of lichenan. The affinity between the CBM-BLG and a "low-molecular weight" β-glucan (20 kDa) from barley was determined to have $K_d \approx 10^{-6}$ M, but we could not

establish how this affinity level compares with that of CBM-BLG and the insoluble lichenan. The affinity, however, of the CBM moiety in the CBM-BLG is reported to be similar for β-glucan with β-(1,3),(1,4)-mixed linkage and lichenan as the CBM in both cases targets the mixed linkage structures [244]. Hence, we expect the affinity of CBM for the insoluble residue of lichenan to be either within the same order of magnitude as the affinity to the soluble fraction of lichenan or barley β-glucans, or even higher due to the decrease in the entropic penalty of binding to the rigid carbohydrate chains in the insoluble fraction. The finding of a moderately higher affinity of the CBM-BLG towards β-glucan (roughly one order of magnitude) than observed for the CBM domain alone, is remarkable and may be explained by a higher degree of freedom of the immobilized CBM-BLG as compared to CBM alone. In other words, immobilization of the fusion protein offers increased sites of covalent attachment to the surface of the chip, enabling some of the protein being attached to the chip surface via the BLG moiety of the fusion, hence leaving the CBM moiety accessible and possibly with a higher degree of freedom to optimize interactions to the polysaccharide analyte as compared to the direct immobilization of the isolated CBM. An alternative explanation may be that the BLG part of the CBM-BLG enhances the affinity by forming weak associations to the carbohydrate molecule. These experiment also indicate that the CBM module in the CBM-BLG is fully functional and that it mediates binding to lichenan and barley β-glucan with similar affinities as in the isolated form, which has a K_d for β-glucan of $3.7x10^{-6}$ M [244].

This study is the first to show that a (1,3),(1,4)-mixed linkage β-glucan holds adjuvant properties in terms of enhancing an antigen-specific antibody response when administered in a non-covalently bound form together with the antigen. Previous studies have demonstrated adjuvant effects of β-glucans consisting of β-(1,3) or β-(1,3),(1,6)-mixed linkage structures [237, 242, 243], and this was independent of the size of the glucan and whether this β-glucan was purified from a natural source [243] or synthetically prepared [53], thus excluding the interference of contaminants. The binding of these structures to the CLRs dectin-1 and CR3 is well established [28], and experiments that target certain CLRs with specific antibodies conjugated to proteins, have shown an increased antigen uptake by APCs without activating signaling pathways in the APCs [173]. β-glucans from barley and lichenan have in a binding study with dectin-1 transfected cells been observed to bind to dectin-1 with an affinity similar to that of curdlan; a β-(1,3)-glucan [233]. Accordingly, we find it likely that β-(1,3),(1,4)-glucans act through the same receptors as β-(1,3)- and β-(1,3),(1,6)-glucans.

We have previously compared the immunomodulatory activity of β-glucans from lichenan and barley *in vitro* and found that lichenan attained increased modulatory activity in DC than barley β-glucan [207]. Lichenan contains more β-(1,3) elements than barley β-glucan [192]. This supports that lichenan binds specific CLRs through the β-(1,3) structures, and to the same receptors as β-glucans from yeast and curdlan, agreeing with the fact that although weaker in its effects, lichenan and barley β-glucans induce the same type of responses in DCs as do β-glucans from yeast and curdlan.

Comparison of the antigen-specific antibody titers with the DC profiles upon stimulation with lichenan and LPS, revealed that lichenan *per se* was able to stimulate maturation of DCs as indicated by a moderate up-regulation of MHC class II and CD86, although the CD86 up-regulation was lower than in DCs stimulated with LPS that additionally showed up-regulation of CD40 and CD80. CD80 and CD86 have been shown to modulate naïve $CD4^+$ polarization towards Th1/Th2 differentiation. CD80 favors Th1 differentiation, while CD86 augments Th2 cell responses [246], and a strong CD40 up-regulation has previously been associated with a Th1 cell polarization [247]. Hence, the DC profiles obtained upon stimulation with lichenan and LPS, respectively, correspond well with the antibody isotype responses elicited towards BLG; with the use of lichenan inducing primarily an IgG1 response, and with LPS giving rise to both an IgG1 and IgG2a response in the BALB/c mice. However, the capacity of lichenan to suppress LPS-induced IL-12p70 production and CD40 and CD80 display, related to Th1 generation, was not demonstrated in the immunization experiment. LPS induced a strong response towards BLG, but no difference was observed between responses from the groups +/- lichenan, indicating that lichenan was incapable of modulating the LPS-induced response *in vivo*. Even more surprising was the enhanced IgG2a response obtained when co-immunizing with CBM-BLG mixed with lichenan and LPS as compared to the response pattern induced by CBM-BLG and LPS alone. This Th1-polarizing property of lichenan when associated to CBM-BLG is somehow in contrast to our DC results, and also to the immunomodulatory effects of lichenan previously reported by others in an experimental arthritis study [240], and we still lack a sound explanation for these findings.

Of note, the general total level of BLG antibodies was higher after injection with BLG alone than after injection with CBM-BLG, but these variations were not statistically significant. To exclude the presence of any stimulating contaminants in the two recombinant protein preparations, we tested if the CBM-BLG or the BLG *per se* could mature DCs. None of the proteins induced pro-

inflammatory cytokine production in DCs (data not shown), hence excluding the presence of an immunostimulating component in the antigen preparations that may account for the larger variation in BLG-specific IgG2a/IgG1 antibody levels in mice injected with BLG alone as in contrast to those injected with CBM-BLG. Moreover, when BLG was used as antigen, immunization with LPS +/- lichenan resulted in a lower BLG-specific IgG1 and IgG2a titer than when the CBM-BLG and LPS +/- lichenan were injected. This indicated that the CBM-BLG may have some affinity for LPS, which may, in parallel to lichenan, enhance the adjuvant activity of LPS. An alternative explanation may be that the increased molecular weight of CBM-BLG, as opposed to BLG, may increase the immunogenicity of the BLG moiety.

In conclusion, we have demonstrated that the plant-derived β-(1,3),(1,4)-glucan lichenan possesses adjuvant properties and induces an antigen-specific IgG response consisting of both IgG1 and IgG2a isotype antibodies when present non-covalently coupled to a protein antigen. When co-injecting lichenan and antigen together with LPS, a further increment in antigen-specific LPS-induced IgG2a responses were observed. This Th1-polarizing property of lichenan was however in contrast to the modulation of LPS-induced DC maturation observed with lichenan. The results may contribute to increase our knowledge into immunoregulatory properties of lichenan, and the influence of antigen/adjuvant associations for induction of antigen-specific antibody responses.

CHAPTER 6

SUMMARY AND CONCLUSION

The purpose of the present chapter is to give a short summary of the main conclusions from the experiments conducted during the thesis period.

In the past decade there has been an increased focus on the immunoregulatory capacity of non-starch polysaccharides (NSP). However, the knowledge regarding this property amongst the NSPs is limited. By far, we still lack insight into the impact of the structure and size of NSPs for the capacity to affect immune responses. In chapter 4, we therefore examined the structural and molecular basis of NSPs for immunoregulatory capacity in DCs. A large panel of assorted NSPs was screened for their ability to modulate cytokine production in TLR4-triggered DCs, using a model of murine bone marrow derived DCs (BMDC). The chosen NSPs were selected to cover polysaccharides both of microbial and plant origin in different structural categories. Some of the NSPs have previously been described as having immunoregulatory activities, whereas other of the NSPs were not formerly reported to hold modulatory properties, although they possess structural familiarities to potent NSPs. Our main focus was on plant polysaccharides that are present in foods, used as food ingredients, or has the potential to be modulatory due to health-promoting attributes.

The immunomodulatory activities observed upon DC stimulation with NSPs concomitant with the TLR4-ligand LPS was balanced towards a high IL-10 production with very low level IL-12p70 secretion. Although limited in its informative value, this DC signature suggests priming towards a tolerogenic milieu, which is involved in Treg subset generation. The NSPs modulated further more the expression of surface markers on DCs. The most potent NSPs were able to up-regulate CD86 *per se*. More diverse modulatory properties were seen amongst the β-glucans upon simultaneous addition of LPS; resulting in a DC phenotype with reduced levels of CD40 and CD80, and enhanced CD86 surface display. CD40 and CD80 are co-stimulatory molecules associated with generation of the Th1 subset, while the role of CD86 for differentiation of T cell subsets is presently unknown. All together the NSPs showed divergent capacities to regulate the DCs phenotype. The potent immunoregulatory NSPs induced a common phenotype in DC that suggests suppression of the Th1-polarizing potency of DCs and a tendency to induce a Treg-promoting phenotype.

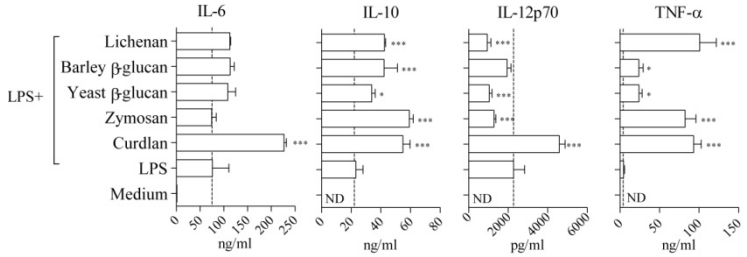
Amongst the various NSPs tested, the DC immunomodulatory activities were primarily found within the group of β-glucans, and as well the galactomannan guar gum. Even among the β-glucans, a certain variation in the immunomodulatory potential was present. β-glucans of microbial sources were found to hold the highest potential compared to those derived from plants. Not only the structure of NSPs was essential, the size of the β-glucan seemed as well to be important for the immunoregulatory properties. However it was not possible to obtain a clear relation between size and responses from our data, as different samples of roughly equal sizes gave non-comparable results. These samples were from different supplier, which could suggest that exstraction methods and subsequent handling of the NSP sample may influence their immunomodulatory capacity. Further studies need to be completed to find the exact correlation between size of the NSPs and their.

NSPs from various sources may contain a variety of different TLR-ligands or other stimulating compounds in varying amounts giving rise to variable effects in DCs, but due to the nature of the NSPs it is not possible to purify for unknown microbial TLR-ligand contaminants. For instance removal of the TLR-ligand LPS from the β-(1,3)-glucan curdlan by available methodologies was impossible due to the insoluble nature of curdlan (LPS-removing columns are clogged up). Another possibility for neutralizing LPS would have been addition of the LPS-binding molecule polymyxin B (PMB) prior to the stimulation. However this was not feasible, as the necessary concentration of PMB (50 ug/mL) affects the production of cytokines from DCs [248].

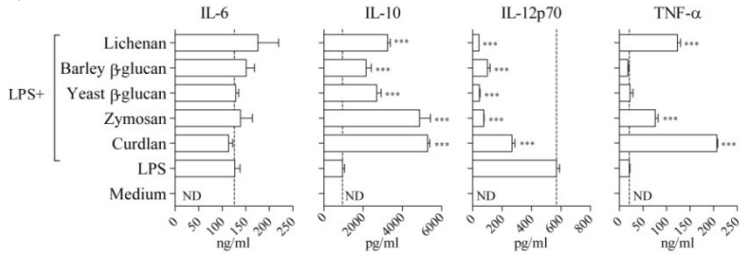
It would have been preferable to perform the screening of the immunoregulatory effect of NSPs in a human DC model instead of the murine BMDC, but since it was not possible to generate enough cells without having donor variability by use of human monocyte derived DC (moDC), the murine DC model was employed. It has previously been shown that human moDC and murine BMDC responses are very similar [185, 249]. A small comparison of the two DC types response against selected NSPs, confirmed that these NSPs virtually induced comparable cytokine responses in both types of DCs, except for curdlan that triggered a response pattern unlike the other β-glucans (Fig. 1). This may be promoted by the presence of a relative large amount of LPS in the curdlan preparation, or it could be due to the presence of other CLR-triggering ligands in any of the β-glucan preparations. One explanation for the differences in response patterns in curdlan-treated

human and mouse DC could be that the curdlan-LPS is more potent in generating a response in human DCs as compared to murine DCs.

A)



B)



C)

		IL-6 (neutral)		IL-10 (up-reg.)		IL-12p70 (down-reg.)		TNF-α (up-reg.)	
		h	m	h	m	h	m	h	m
LPS +	Lichenan	+	+	+	+	+	+	+	+
	Barley β-glucan	+	+	+	+	-	+	+	-
	Yeast β-glucan	+	+	+	+	+	+	+	-
	Zymosan	+	+	+	+	+	+	+	+
	Curdlan*	-	+	+	+	-	+	+	+

All samples are compared to LPS *per se.*

* Curdlan strongly up-regulates hIL-12.

Figure 4. Comparison of the effect of NSPs on LPS-induced cytokine production in human and murine DC.
Data represents the levels of cytokines in culture supernatants from (A) human monocyte-derived DC and (B) murine bone marrow derived DC upon culturing for 18 h with the indicated NSP (100 μg/ml) in the presence of LPS (1 μg/ml) or with LPS alone. The dashed line represents cytokine production from LPS-treated DCs. The levels of cytokines were determined by ELISA. Data are mean + SD, and represents 3 different experiments. Differences between dual-treated DCs as compared to LPS-treated DCs were tested by one-way ANOVA and the Tukey post-test. $P < 0.05$, *, $P < 0.01$, **, $P < 0.001$. ***. (C) Comparison of the human (h) and murine (m) data. + corresponds to similar regulatory profiles, and – to divergent effects of NSPs in the two model systems.

Another focus was to examine in detail the DC phenotype induced by β-glucans of different origins. In chapter 5, we describe the distinct regulations of TLR-triggered phenotypes in human moDCs by diverse β-glucans, and designate a general β-glucan-mediated phenotype in TLR4-triggered DCs by measuring an array of cytokines, chemokines and surface marker expression levels. Furthermore, we look at the activation pattern of the CLRs Dectin-1, DC-SIGN and Mannose receptor (MR) upon interaction with the different β-glucans.

All β-glucans were able to modulate the phenotype of the TLR-primed human DCs. In general induced the β-glucans a phenotype characteristic by reduced production of CXCL10 in conjunction with enhanced production of CCL4, CXCL8, IL-1β, IL-10, IL-2, and TNF-α. Dependent of the origin of the β-glucans, varied levels of IL-6, IL-12p70, IL-23 and CCL17 were obtained. These

cytokine and chemokine profiles suggest that the β-glucan- and TLR-primed DCs have the potential to induce both a Treg and Th17 responses. The DCs were dispersed into two subsets: one with low level CD40 and CD86, and the other with high level CD86 and intermediate CD40 display, these tow subsets may explain the secreation of both Treg and Th17-inducing components. Curdlan was the only β-glucan observed to augment the IL-12p70 production and at the same time induce substantial levels of IL-23. One explanation for this may be that the LPS contamination enhanced the levels of pro-inflammatory cytokines, but another reason for the divergent response from curdlan may rely on the lack of CLR-regulating ligands that may be present in some of the other β-glucan preparations, such as the mannan detected in zymosan, yeast β-glucan and lichenan. Activation of MR is previously reported to induce IL-12p70 suppression and enhancement of IL-10 production in a TLR-triggering environment [83]. To address the exact T-cell polarizing effects of the β-glucans, further experiments like co-cultivating of the β-glucan-modulated DCs with naïve T cells will have to be conducted.

The β-glucan-induced DC phenotypes were observed to be mediated via interaction with the CLRs, as reduced expression levels of the CLRs: Dectin-1, DC-SIGN and MR on the β-glucan-treated TLR-triggered human moDC were observed. The individual CLRs were regulated in a ligand-receptor specific manner. Dectin-1 was the main receptor for β-glucan, as the expression was down-regulated by all β-glucans. Both MR and DC-SIGN are suggested to be receptors for mannose- and fucose containing compounds. Nevertheless, some of the β-glucans were observed to down-regulate the display of MR and DC-SIGN which may be due to the presence of mannose-containing compounds in these products. The regulatory pattern induced by the different CLRs also indicated that the ligands for MR and DC-SIGN are more similar than the one for Dectin-1. Presently, we do not have data that confirms involvement of other CLRs, but we find it plausible that other receptors are contributing to the regulatory phenotype in DCs.

The aim of the final study in this thesis was to test whether the immunomodulatory effects observed in the described *in vitro* systems could be utilized *in vivo*. The objective of the study presented in chapter 6 was to gain insight into the *in vivo* modulatory properties of lichenan, as a model for β-glucans. Specific focus was on the importance of a complex formation between the antigen and lichenan. The approach was to examine the potential of lichenan to engage as an adjuvant and

induce an antigen-specific response towards an antigen when mixed and co-injected together, as compared to being non-covalently associated to the antigen in a fusion protein (CBM-BLG) consisting of antigen (BLG) and a β-glucan-binding domain (CBM) from *Clostridium thermocellum*. In addition, we studied if lichenan could mediate modulation of the immune response against the antigen when co-injected with the TLR-4 ligand LPS to trigger a Th1-polarized immune responses.

In order to achieve a tight association between the antigen and lichenan, a fusion protein with β-glucan binding capacity was constructed. The antigen BLG was fused with CBM via overlap PCR and expressed in *Pichia Pastoris*. The purified proteins retained its β-glucan binding capacity, which was confirmed via surface plasmon resonance analysis and an adsorption assay.

In order to test *in vivo* effects of lichenan, CBM-BLG or BLG alone were mixed either with lichenan, lipopolysaccharide (LPS) or both, and injected intraperitoneally into mice. BLG-specific antibody titers measured in blood samples revealed that lichenan caused a significant increase in the IgG antibody response, but only if lichenan was combined with CBM-BLG. This indicated that the complex formation between antigen and the β-glucan could enhance the response against the antigen. The specific IgG isotypes IgG1 (Th2) and IgG2a (Th1) were also measured to describe Th1/Th2 polarizing properties. The ratio of IgG2a/IgG was enhanced when lichenan was mixed with CBM-BLG, and further improved when LPS was added, thus suggesting a Th1-polarizing potential of lichenan when associated with the antigen. This Th1-polarizing property of lichenan when associated to CBM-BLG was in contrast to the *in vitro* DC results reported in chapter 4. The reason was, however, not identified.

Collective main conclusions from the present studies:

- β-glucans and the galactomannan guar gum induce modulation of a TLR-triggered phenotype in DC, with the exact structure of the non-starch polysaccharides being important for the activity. The size may be another factor of specific importance in relation to the immunoregulatory properties of the products.
- The NSPs induce a DC phenotype with possible Treg or Th17-promoting properties.
- Activation of CLRs largely depends on the nature of the receptor and the ligand. Dectin-1 is activated by β-glucans of diverse origins, and with different molecular structures.

- The β-glucan lichenan improves the Th1-adjuvant properties of LPS, and exhibits antigen-specific Th1/Th2-polarizing adjuvant activity when non-covalently associated to antigen.

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